PROCEEDINGS
OF A

FORENSIC SCIENCE SYMPOSIUM ON THE ANALYSIS OF SEXUAL ASSAULT EVIDENCE

CO-HOST
FEDERAL BUREAU OF INVESTIGATION

CO-HOST
METRO-DADE POLICE DEPARTMENT
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Foreword

On July 6–8, 1983, the Federal Bureau of Investigation and the Metro-Dade Police Department co-hosted a Forensic Science Symposium on the Analysis of Sexual Assault Evidence. The symposium was conducted at the Forensic Science Research and Training Center at Quantico, Virginia, and was attended by 166 scientists representing 125 laboratories from throughout the United States and abroad.

This symposium was designed to bring together representatives from academia, private laboratories and federal, state and local crime laboratories to address mutual concerns in the analysis of evidence relating to sexual assault. From the response of the participants, it was an overwhelming success. The program provided ample opportunities for the introduction of new ideas as well as for the discussion and demonstration of many current practices and techniques.

We hope that the spirit of cooperation and exchange evident at this meeting continues and that this publication serves as a useful reference for practitioners in this rapidly changing forensic discipline.

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Contents

SECTION I
LECTURES

ANATOMY, PHYSIOLOGY AND BIOCHEMISTRY OF THE FEMALE REPRODUCTIVE SYSTEM

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The forensic scientist is presented with many types of trace physical evidence for examination. In cases from sexual assault there are usually vaginal swabs and clothing from the victim and the forensic scientist is asked to identify semen. Identification of vaginal constituents would be sought. As detailed later in this publication there are genetic markers in semen, such as the ABH blood group substances and the polymorphic enzyme phosphoglucomutase which can be used to aid in the individualization of the suspect. These components are also in the vaginal fluid. In many cases semen and vaginal components are mixed and the scientist is asked to explain how these mixtures can influence the interpretation. This chapter is designed to provide basic information necessary to understand normal female anatomy and physiology and thus incorporate this information into the understanding and interpretation of data derived from the analysis of physical evidence from sexual assault.

It would be appropriate at this time to provide a brief overview of the female reproductive system so that the detailed discussions that follow will be more relevant. There are more detailed references that describe female anatomy (Netter 1977) and the physiology and endocrine interrelationships (Goodman 1974; Speroff *et al.* 1978; Williams 1981) of the female. Anatomically, the female reproductive system consists of the ovaries, which serve two functions, the production of hormones and germ cells, and the duct system consisting of the oviduct, uterus, cervix, vagina and vulva. The hormonal control of the female reproductive system is a delicate balance between the hypothalamus and the anterior pituitary within the brain and the ovaries in the abdomen. Chemical messengers, known as hormones, emanate from the hypophythalamus in pulsating fashion in the mature female and stimulate the anterior pituitary to release two hormones, follicle stimulating hormone (FSH) and luteinizing hormone (LH). It is these two hormones, FSH and LH, that stimulate the ovaries to release germ cells (ova) and steroid hormones, the estrogens and progestins. These latter two hormones affect the reproductive tract resulting in menstruation at regular intervals and cyclic changes in cellular morphology of the reproductive tracts, such as the vagina. Interestingly, the hormones of the ovaries feed back on the brain to cause either a positive or negative stimulus, thus influencing the release of FSH and LH.

Sexual Development and Differentiation

Sexual development begins with the union of germ cells, ova from the female and sperm from the male, at the time of fertilization. At this time the genetic sex is established. The germ cells of the female, the ova, contain one half of the genetic information of the parent and since the female is homozygous XX, the ova (being haploid) would only exhibit one sex chromatin, the X. Conversely, the male is the heterogametic sex (XY) and the male germ cells, spermatozoa, will contain either the X or Y chromatin material. At the time of fertilization the X bearing ova and X bearing sperm will combine to form the genetic homozygous XX female. The genetic XY male would result from the union of the X bearing ova and Y bearing sperm. Regardless of the genetic sex, the female XX or male XY, during the early stages of embryonic development it is difficult if not impossible to distinguish the reproductive tract of the fetus as being female or male based upon either gross or microscopic examination.

In point of fact, the tissues necessary to develop into either a female reproductive tract or male reproductive tract are both in place, although in a rudimentary form. Differentiation into the reproductive tract system that resembles the phenotypic sex and thus matches the genotypic sex is dependent upon a critical timing of the fetal gonads to signal these rudimentary fetal tissues to differentiate into the correct tissues. Actually, evidence indicates that a requirement for the undifferentiated tissue to develop into the male type requires fetal testicular substances, one to stimulate the male tissues and the other to inhibit the female tissues, otherwise the female reproductive system will develop (Jost 1971).

Gonadal Differentiation

Both the male and female reproductive systems develop from specific embryological tissues and in many instances share a common origin for some specific tissues (Figure 1) The ovaries of the female and the testes of the male both begin as indifferent gonads where they are morphologically indistinguishable from one another until approximately 42 days of gestation. The primative gonads are derived from proliferation of the mesodermal coelomic epithelium, mesenchymal cell masses on the urogenital ridge and from mesonephric elements. Primordial germ cells have seeded the undifferentiated gonad by about 42 days of gestational age. In the female the gonads differentiate into the ovaries and these primordial germ cells will become oogonia whereas in the male the gonads become the testes and the germ cells become the spermatogonia. Although the testis begins to organize at about 45 days, under the influence of the HÇY antigen, the ovary does not differentiate until three months of gestational age. It has been estimated that 1,000 to 2,000 primordial germ cells migrate to the human embryonic ovary and give rise to a peak population of about six million germ cells by the end of the fifth month in both ovaries (Baker 1963). The formation of germinal cells stops by the seventh month of gestation and no additional germ cells are added to the ovaries. In fact, at the time of birth there begins a rapid decline in the number of cells available for potential development so that by the time of puberty there are between 40,000 and 400,000 oocytes. Then between puberty and menopause the 8,000 surviving oocytes undergo additional partial maturation, however, only 400 to 500 ever become potential ova.

Ductal Differentiation

The tissues associated with specific portions of the female reproductive system (tract) arise from the mesial aspects of genital thickening on the posterior surface of the embryonic body cavity. The internal genital tracts in the two sexes develop from two different sets of tracts, the Wolffian and Mullerian ducts, collectively called the discrete primordia (Figure 1). These temporarily coexist until the third month of fetal life, regardless of genetic sex. As mentioned previously, if the embryo is a male, the testis begins to organize earlier (45 days) than the ovaries would develop if the embryo were female (3 months). The fetal testis secretes a glycoprotein, Mullerian inhibiting substance, which causes regression of the Mullerian ducts, ultimately resulting in their disappearance (Wilson *et al.* 1981). Within a few days, the internal genital tracts of the male begin to develop (termed virilization) and ultimately the Wolffian duct completes its development In the female embryo, the Wolffian ducts regress and the Mullerian ducts develop into the fallopian tubes, the uterus and upper portions of the vagina. Interestingly, Mullerian duct development is not contingent on the presence of an ovary since equally good development of the uterus and oviduct will take place if no ovary is present.

External Genitalia Differentiation

In contrast to the internal genitalia which develop from separate duct systems in males and females as just described, the external genitalia of both sexes develop from a common anlage. At the eighth fetal week the differentiation into female or male external genitalia begins. The lower portion of the vagina, labia minora and majora and the body of the clitoris have analogous tissues in the male (Figure 2). The clitoris is the homologous tissue to the penis and derives from the genital tubercle by the 14th week of gestation while the labia majora and minora in the female develop from the labio-scrotal swellings and urethral folds respectively, and are homologous in the male to the scrotum and corpus spongiosum, respectively. The urogenital sinus makes contact with the fused Mullerian duct and forms the lower portion of the vagina. The Skene's glands and Bartholin's gland develop in the female urogenital sinus and have homologous origins to the prostate and bulbourethral glands (Cowper's) in the male.

In the male, development of the external genitalia is influenced by androgens derived from the embryonic testes and begins shortly after the onset of virilization of the Wolffian ducts. Although the external genitalia of the female enlarge, there is little change from the indifferent state. In contrast, in the male, the genital folds elongate and fuse to form the penis and male urethra. The fusion of the urogenital swellings on each side of the urethral orifice form the scrotum that serves as the receptacle for the testes.

There are instances when the female fetus is exposed to hormones with androgen activity, whether of fetal or maternal origin, resulting in androgen-induced forms of human female pseudohermaphrodism. Generally, the urogenital sinus and external genitalia are developed along masculine lines (Grumbach and Ducharme 1960). However, the most common cause of female masculinization is congenital adrenal hyperplasia in females. This condition results from an inherited deficiency of one of several enzymes necessary for normal adrenal steroid synthesis and results in excessive secretion of androgenic steroids by the fetal adrenal gland (New *et al.* 1981).

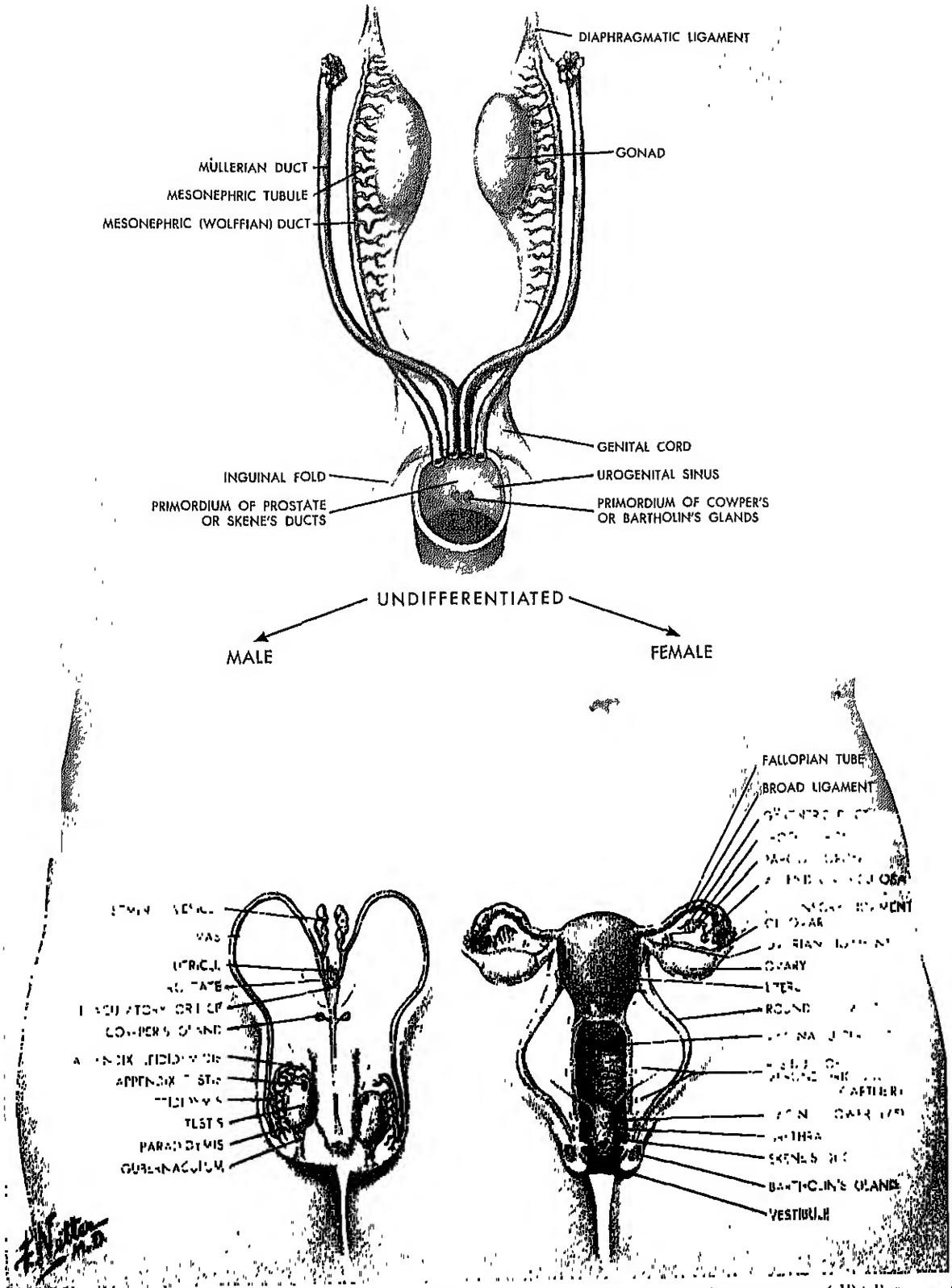

Figure 1. The anatomic structures of the undifferentiated reproductive system and its development. (Copyright 1965, CIBA Pharmaceutical Company, Division of CIBA-GEIGY Corporation. Reprinted with permission from THE CIBA COLLECTION OF MEDICAL ILLUSTRATIONS, illustrated by Frank H. Netter, M.D. All rights reserved.)

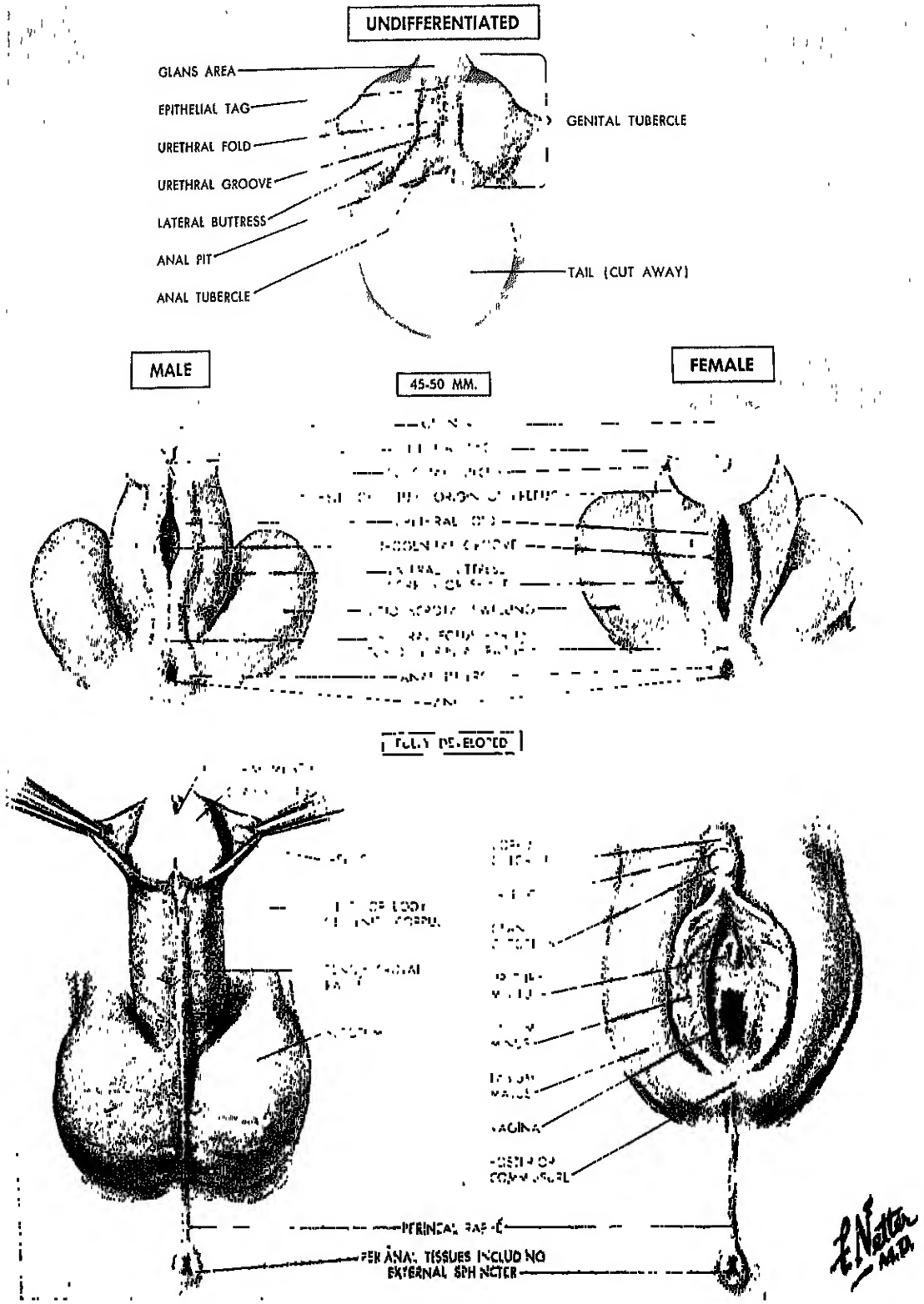

Figure 2. The external genitalia of the female and male. (Copyright 1965, CIBA Pharmaceutical Company, Division of CIBA-GEIGY Corpora tion Reprinted with permission from THE CIBA COLLECTION OF MEDICAL ILLUSTRATIONS, illustrated by Frank H. Netter, M.D. All rights reserved).

Mature Female Anatomy

The female reproductive tract is oriented in the lower abdomen and consists of the vulva, vagina, cervix, uterus, oviduct and the ovaries.

The vagina (Latin for sheath) is a tubular structure made up of three layers; stratified squamous epithelium, muscular and connective tissue. The vagina extends from the vestibule of the vulva to the cervix.

The cervix (Figure 3), which technically is part of the uterus, is approximately 4 cm in length and 3 cm in diameter. One half of its total length projects into the vagina and is called the portio vaginalis; the rest of the cervix is imbedded within the vaginal wall and is continuous with the body of the uterus. The bulk of the cervix is connective tissue and contains high columnar mucus secreting epithelial cells.

The body of the uterus is anchored in the pelvis by a series of bands of connective tissue or ligaments (Figure 3). The body of the uterus is shaped like an inverted pear and is 4 to 7 cm in length and 4 to 7 cm wide at its widest point. The thick wall of the uterus is made up of three layers; an inner epithelium (endometrium), a large complex of smooth muscle (myometrium) and an outer connective tissue (serosal) layer.

The oviduct (Fallopian tube, uterine tube) is about 12 cm long and 3 to 5 mm in diameter. One end is attached to the body of the uterus and the other end opens near the ovary in the abdominal cavity (Figure 3). A narrow canal is present throughout the entire length of the tube. The inner epithelium contains secretory and ciliated cells. The opening of the oviduct into the abdominal cavity at the ovarian end, the fimbria, is lined with ciliated epithelial cells, which are important in pick-up and transport of the ova into the oviduct.

The ovaries are ovoid in shape each measuring 4 x 2 x 2 cm and are located anatomically in the vicinity of the fimbria of the oviduct (Figure 3). The ovaries are suspended by peritoneal folds. As depicted in Figure 3, the cross section of the ovary illustrates a number of distinct structures. The follicle has developed from a primary follicle in the germinal epithelium through the influence of FSH and LH. There are a number of stages of growth of the follicle and it finally reaches the stage of Graafian follicle with the ova inside surrounded by follicular fluid. During this time of follicular growth the ovary is producing ever larger quantities of estrogens which have specific stimulatory properties on the reproductive tissues. The next stage of development for the follicle on that ovary would be at the time of ovulation, at which time the Graafian follicle would rupture through the stimulation of LH. As the follicle ruptures the contents of the follicle spill out into the abdomen where the fibrillating cells of the infidibulum cause a wave of activity and draw the ova with its surrounding mass of cells into the oviduct. The ruptured Graafian follicle now fills with blood and is now known as the corpus hemorhagicum. Again under the influence of LH the corpus hemorhagicum now forms the corpus luteum (CL) (Figure 3). The CL produces large quantities of the steroid hormone, progesterone, which stimulates growth and development of the uterus to prepare the uterus for the fertilized ova, in the event fertilization has taken place. In the event fertilization has not taken place or if the fertilized ova does not survive, the CL will regress, producing smaller and smaller quantities of progesterone. The final structure remaining from the original primary follicle that went through full maturation, ovulated, formed the CL and has regressed, is now referred to as the corpus albicans (Figure 3). Thus the complete saga of one primary follicle has been completed. This cycle continues through the reproductive life, beginning at puberty at approximately 13 years of age, and continues every 28 days, interrupted only by pregnancy, until the female approaches menopause at about 50 years of age. At this time the ovary becomes nonresponsive to the hormones from the adenohypophysis and regresses.

Endocrine Regulation of the Menstrual Cycle

The female endocrine system is dynamic, involving hormones of the brain and ovaries that alternately serve in a stimulatory and inhibitory fashion. The lower portion of the brain contains the hypothalamus which synthesizes and releases on a cyclic fashion a number of different polypeptides referred to as releasing factors or releasing hormones. These releasing factors are transported by a specific blood system (the hypothalamic-hypophyseal portal system) to the adenohypophysis (also referred to as the anterior pituitary). The adenohypophysis synthesizes and releases a number of hormones, among which are two glycoproteins, FSH and LH. FSH and LH are classified as gonadotropins because of their stimulatory influence on the gonads.

The menstrual cycle can best be divided into three phases: the follicular phase, ovulation and the luteal phase. During the follicular phase of the cycle an orderly sequence of events takes place culminating in ovulation. This follicular phase occurs over a space of 10 to 14 days and begins with increasing circulating levels of FSH serving to stimulate the growth and development of ovarian follicles. At about the mid-follicular stage (days 7 to 10), there is a significant increase in plasma estrogen. Estradiol is the major

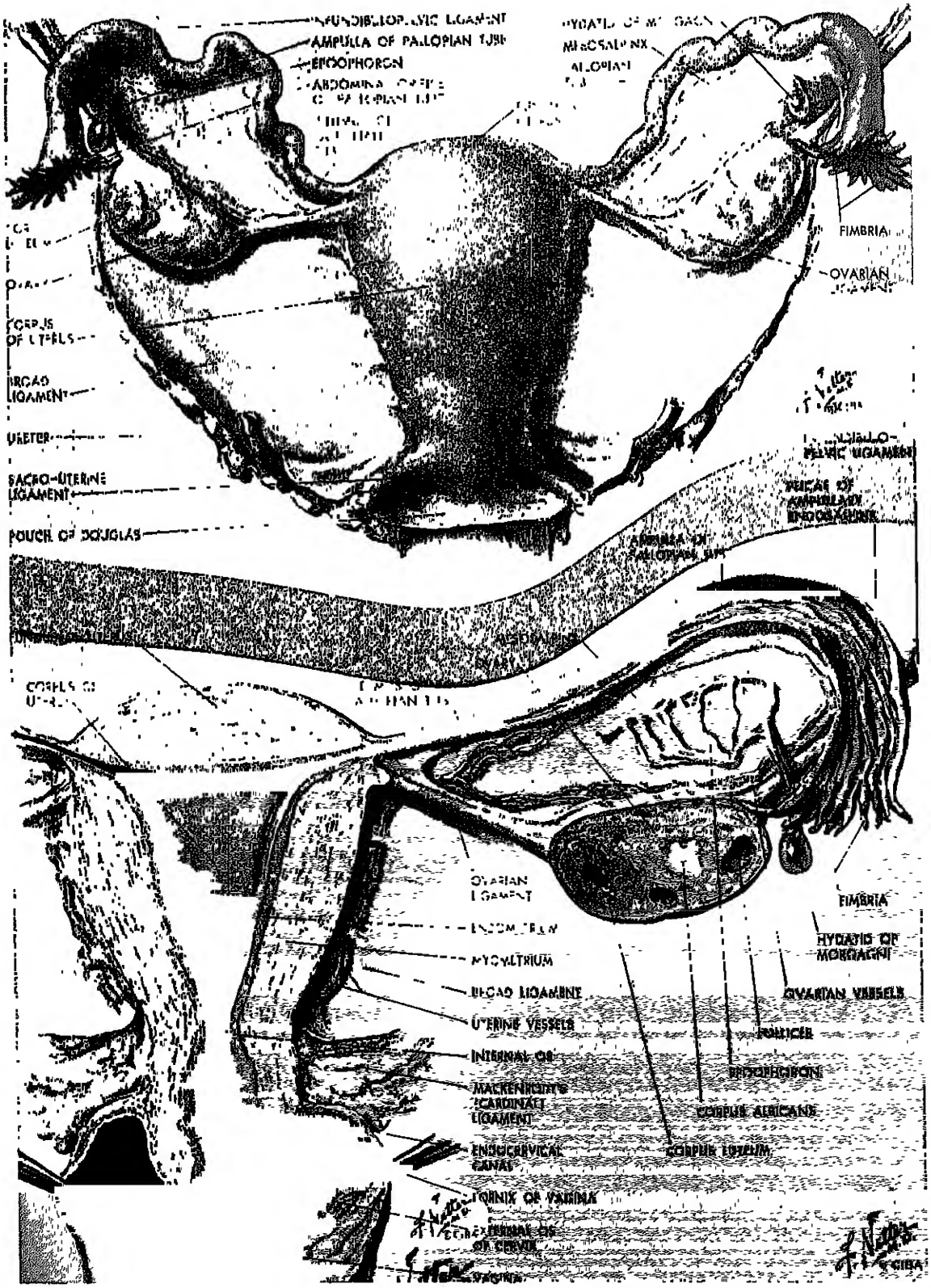

Figure 3. Schematic of the [illegible] female genital organs. (Copyright 1965 CIBA Pharmaceutical Company, Division of CIBA GEIGY Corporation. Reproduced with permission from THE CIBA COLLECTION OF MEDICAL ILLUSTRATIONS, illustrated by Frank H. Netter, M.D. All rights reserved)

estrogen secreted by the ovary of the human and rises from a level of approximately 0.010 ng/ml to 0.025 ng/ml plasma. This increase in circulating estradiol inhibits the release of more FSH but stimulates LH release from the hypothalamo-hypophyseal system. During the late follicular phase (days 10 to 14), also referred to as the preovulatory phase, estradiol continues to rise and reaches a peak (up to 0.20 ng/ml plasma) just before ovulation.

The high level of estradiol at this stage of the cycle stimulates the gonadotropin surge associated with ovulation, a modest rise in FSH and a dramatic peak of LH (100 ng/ml plasma). The high levels of gonadotropins persist for about 24 hours, then decrease during the remainder of the luteal phase of the cycle. The Graafian follicle ruptures within 24 hours of the LH peak and the next phase of the cycle begins.

The luteal phase of the menstrual cycle lasts approximately 14 days, beginning with the formation of the CL (previously described). Plasma progesterone is secreted by the CL and increases to an average of 10 ng/ml with a level of 3 ng/ml serving as reliable evidence of ovulation (Speroff *et al.* 1978). Beginning about 10 to 12 days after ovulation, the corpus luteum enters into a stage of regression noted by a decrease of progesterone. During this period large follicles are once again seen within the ovaries, thus the stage for follicular development of the next cycle has already been set. As progesterone plasma levels reach a base line of 0.2 ng/ml due to the complete regression of the CL, the endometrial lining of the uterus sheds and menstruation begins. Menstruation (menses or the period) is, of course, characterized by the appearance of non-clotted blood in the vagina and will be discussed in more detail later in this chapter.

Unless pregnancy occurs the degeneration of the CL is inevitable. With pregnancy, however, the survival of the CL is prolonged by the appearance of a new gonadotropin, human chorionic gonadotropin (HCG). HCG first appears 8 to 12 days after ovulation, prevents luteal regression and maintains progesterone production by the CL until approximately the 10th week of pregnancy. At this time the placenta is capable of sufficient steroid production to maintain pregnancy so plasma HCG levels decline. The confirmation of pregnancy is the detection of HCG in blood or urine.

To summarize, the normal human menstrual cycle is one of recycling, unless interrupted by pregnancy, of approximately 28 days in duration. From a reference standpoint, the menstrual cycle begins with menses (day 0 begins with bleeding), lasting 3 to 5 days. The follicles respond to rising levels of FSH by growing and releasing estradiol. Ovulation (at day 14 to 15 of the cycle) is triggered by the estradiol induced gonadotropin peak. Under the influence of LH the CL develops and releases progesterone for about 12 days. The CL regresses, plasma progesterone declines and menstruation begins.

Both the levels of the different hormones and the sequence in which they appear are crucial for the coordination of this complex system. It is interesting to note that the steroids progesterone and estradiol can alternately stimulate and inhibit gonadotropin release from the adenohypophysis by influencing the hypothalamus and adenohypophysis. In fact, the gonadotropins themselves, as they are released from the adenohypophysis, serve to inhibit the hypothalamus from releasing the gonadotropin releasing factors.

Menarche, Menstruation and Menopause (Climacteric)

There are a number of changes that occur as the female enters puberty (age 11 to 13 years) which includes breast bud development, then pubic and axillary hair growth, followed by acceleration of growth and finally menarche, the onset of menses. Menses begins after the peak growth in height has occurred. These changes are precipitated by the increase in circulating levels of estrogens and progesterone produced by the ovary and the increase in sensitivity of the hypothalamic centers to these gonadal steroids. From the time shortly after birth to the onset of puberty the hypothalamic centers are generally refractory to gonadal steroids. Early menstrual cycles are generally anovulatory and therefore irregular. This lasts for 12 to 18 months until adult levels of gonadotropins and gonadal steroids are reached. Thereafter menses occurs on a regular basis approximately every 28 days.

The series of endometrial events associated with the menstrual cycle and characterized by the overt indication of the cyclic nature of reproductive function in the mature human female has been well defined. This is, of course, menses, the periodic discharge of blood from the vagina, a result of sloughing off of the fully developed progestational endometrium.

A subhuman primate, the rhesus monkey, was used to study the morphological basis for menstrual bleeding and the findings were later confirmed in human studies. Markee (1948) transplanted endometrium to the anterior chamber of the eye of the monkey and was able to determine the morphological basis for menstrual bleeding as associated with the changes of the ovary. Subsequent work by Bartelmez (1957) in the rhesus monkey and Rock *et al.* (1959) in the human confirmed these earlier observations. Ferenczy and Guralnick (1979) have reviewed the kinetics of menstrual endometrium in the human.

The actual mechanism for menstruation begins with thickening of the endometrium during the follicular

phase of the cycle. Under the influence of ovarian estrogens, uterine glands elongate and spiral arteries grow to supply this thickened endometrium. During the luteal phase of the cycle, there is further thickening of the endometrium, marked growth of coiled arteries and the complexity of the uterine glands are increased. With decline in circulating levels of progesterone, due to atrophy of the corpus luteum, there is reduction in endometrial thickness. A few hours before the onset of the flow of blood there is vasoconstriction of a portion of the spiral arteries adjacent to the myometrium. Then with this endometrial shrinkage there is loss of blood from the endometrium. The endometrium begins repair almost immediately under the influence of estrogen and growth of the endometrium begins anew.

Although this event occurs on the average every 28 days, there are wide variations in frequency and intensity. Menstruation typically lasts 3 to 6 days while the average volume of blood loss ranges from 20 to 30 ml per day (Beller and Schweppe 1979).

In addition to the cellular components (erythrocytes, platelets, eosinophils, basophils, neutrophils, lymphocytes and monocytes) menstrual blood consists of plasmin, trypsin-like and other proteolytic enzymes of undetermined nature (Ebert *et al.* 1979). Ebert and coworkers (1979) also reported $\alpha 2$ macroglobulin and $\alpha 1$ trypsin inhibitor were present although considerably below peripheral plasma concentrations. They found a high concentration of fibrin(ogen) degradation products but could provide no evidence for clottable fibrinogen. Although they found clots in the vagina of some subjects these were discovered to be aggregates of erythrocytes and not coagulation clots. Thus Ebert *et al.* (1979) postulate that during menstruation fibrinogen is dissolved rapidly by various proteolytic enzymes, among them primarily plasmin, which may be activated by plasminogen activators.

Menstruation and all the associated hormonal and reproductive tissue changes just described will occur at these regular intervals in the normal female, interrupted only by pregnancy, for almost three decades. Then, as the female enters her fifth decade of life, the frequency of ovulation diminishes. There is a reduction in the amount of estrogen being produced, menstruation becomes irregular and normal reproductive function ends. The period of waning ovarian function is called climacteric and can last several years but generally ends at about age 50 at which point the female has reached menopause. During the three decades of normal ovarian function, the estrogen dependent tissues have been maintained and these now begin to atrophy, due to the lower levels of estrogen. The plasma estradiol is now at a baseline level (0.010 ng/ml) and is no longer capable of exerting an inhibitory influence on the hypothalamic centers. Consequently, plasma FSH and LH increase dramatically.

Amenorrhea

The absence or cessation of menstrual bleeding is termed amenorrhea and results from a number of physiologic and mechanical aberrations. Primary amenorrhea is a failure of menarche to occur by the age of 18 years. This disorder results from fetal errors in gonadal, gonaductal and genital development in about 60 percent of the cases while the remaining 40 percent of the women have primary amenorrhea due to causes such as hypogonadotropic states, other endocrinopathies, sclerocysticovaries and endometrial synechiae (Williams 1981).

Secondary amenorrhea is the absence of menses for a period of three months in females who have previously menstruated. Aside from a number of pathophysiologic causes of secondary amenorrhea, including pregnancy and menopause. Pathophysiologic causes range from normal, to absence of to excess ovarian function. As previously discussed, the ovaries secrete estradiol and progesterone which exert an effect on uterine endometrium and on the hypothalamic-hypophyseal axis. Alteration in the normal levels of these steroids would adversely influence either the endometrium and/or the hypothalamic-hypophyseal axis resulting in secondary amenorrhea. Excess androgen production (testosterone and androstenedione) by the ovaries or adrenals also results in secondary amenorrhea and is frequently characterized by hirsutism and virilism (Osborn and Yannone 1971; Abraham and Chakmakjian 1974).

Fertilization and Pregnancy

In the human, sperm are deposited into the vagina at time of coitus. Transport of sperm through the cervix into the uterus and up into the oviduct begins immediately. Sperm have been located in the oviduct five minutes after deposition in the vagina (Settlage *et al.* 1973). Although millions of sperm are deposited in the vagina only a hundred or so actually are found in the oviduct at the time of fertilization. The cervix appears to play a significant role in accumulating sperm in the cervical mucus and then releasing them into the uterus for several hours.

Although sperm reach the oviduct within a few minutes after coitus, sperm survive in a viable condition necessary for fertilization for hours and are found in the reproductive tract for a few days. Ahlgren (1975) has reviewed transport to survival of sperm in the oviduct and concluded survival of 24 hours is normal. In fact sperm were found in the peritoneal fluid in 5 of

14 females within 24 hours of coitus (Horne and Thibault 1962).

At the time of ovulation the ovum (egg) and its surrounding mass of cumulus cells are transported into the oviduct. The mechanism for ovum pickup from the ovary or peritoneal cavity involves the ciliary activity of the oviductal epithelium and muscular contractions of the oviduct. Transport through the oviduct then begins at the time of ovulation and ends approximately three days later as the egg passes into the uterus (Croxatto and Ortiz 1975; Mastroianni and Komins 1975)). Once in the oviduct the cumulus cells are gradually dispersed. The fluids of the oviduct, provided through the appropriate sequence of hormonal events, create an ideal environment for both the egg and sperm. The composition of the oviduct fluid has been studied extensively (Moghissi 1970; Hamner 1973; Lippes *et al.* 1981). Should fertilization take place, it would occur in the oviduct and the first cleavage would take place here prior to transport through the isthmus to the uterus where implantation would take place.

There are a number of physiological and endocrine events associated with pregnancy in the human. As the blastocyst increases in size there are insufficient nutrients in the uterine environment to sustain growth so a placenta forms. The placenta provides a link between the maternal and fetal circulation and allows for the physiological exchange of molecules between fetal and maternal tissues necessary for growth. The placenta has limited permeability thereby creating a barrier to large materials including bacteria. As the fetus grows more nutrients are required and the maternal circulation increases 1 to 1.5 L to furnish these additional nutrients and to remove metabolic waste from the fetus.

The placenta also produces a number of hormones that sustain pregnancy and contribute to the growth and development of the fetus. The chorionic cells of the developing placenta begin to secrete HCG, a glycoprotein. HCG is a luteotropic hormone, that is, it saves the corpus luteum from regressing and stimulates it to secrete progesterone. Thus, progesterone levels are maintained and continue to promote an ideal uterine environment for the developing conceptus, including preventing endometrial shedding (menses) and inhibiting the hypothalamus from initiating events associated with follicular development and the onset of a new ovarian cycle. The urinary and peripheral blood levels of HCG increase markedly in the early weeks of pregnancy and the detection of HCG in blood or urine is used as a test for pregnancy. The concentration of HCG peaks in the 10th week of pregnancy at approximately 100 IU/ml plasma then declines by the 17th week of pregnancy to about 20 IU/ml plasma for the remainder of pregnancy and disappears after parturition.

Additional placental hormones are human placental lactogen (HPL), progesterone and estriol, all of which either promote growth and development of the fetus or contribute to mammary development for postpartum lactation. HPL is also a glycoprotein produced by the placenta and promotes mammary growth. Although HPL synthesis begins early in pregnancy, about the same time as HCG, HPL peaks much later, at 32 weeks of pregnancy and remains high for the duration of pregnancy (a secretion rate of 1 gm/day is normal), then disappears within 1 or 2 days after parturition. The placenta begins secreting progesterone in the early stages of pregnancy and soon replaces the corpus luteum as the principal source of progesterone. The placental progesterone rate of production reaches 250 mg/day with plasma levels averaging 150 ng/ml. The placenta also produces the estrogenic steroid estriol which increases as pregnancy progresses. Decreased levels of estriol generally indicate fetal distress in the last trimester of pregnancy.

Parturition is initiated about nine months after fertilization in the human and ends with the delivery of the fetus and placenta with a blood loss of approximately 0.5 L. There are a number of endocrine alterations at the end of pregnancy and several hormones seem to play a role in parturition. Progesterone levels decrease removing their inhibitory role on uterine contractility. Prostaglandin levels increase as does a neurohypophyseal peptide, oxytocin, both of which promote uterine contractions. Additionally, the fetal adrenal produces large amounts of corticosteroids which play a role in parturition.

Lactation begins when the maternal breast, which has developed from long exposure to HPL, prolactin (an adenohypophyseal hormone), progesterone and estrogen, experiences withdrawal of estrogen and progesterone. Thereafter, the hypophyseal hormones oxytocin (for milk let down) and prolactin (for milk synthesis) are responsible for lactation. If postpartum nursing is prolonged, amenorrhea will usually continue for as long as 4 to 6 months and frequently longer. Endocrine events that initiate ovarian cycles will then commence and menses will return.

Vaginal Histology

The histologic morphology of the vaginal mucosa is unique in its response to sex-steroid hormonal stimuli. The cyclic morphological changes in vaginal epithelium and the correlation to the phases of the ovarian cycle were first demonstrated by Stockard and Papa-

in the human.

At sexual maturity three distinct zones may be observed within the squamous epithelium (five zones have been observed via the electron microscope by Burgos and Roig de Vargas-Linares 1978); the basal zone which is in constant epithelial regeneration; the intermediate layer which consists of flat cells with clusters of glycogen; and the superficial layer which contains cells larger in size than the intermediate cells and contains pyknotic nuclei (Figure 4).

The vagina is lined by mucosal membrane consisting of stratified squamous epithelium and when a vaginal swab is taken one would expect to find a number of different cell types depending upon the stage of the menstrual cycle (Figures 5a and 5b). Thus, the vaginal epithelium is influenced by the gonadal hormones. In the early proliferative phase the exfoliated cells are predominant with precornified epithelium characteristic of the basophilic cytoplasm. During the late proliferative phase, when the reproductive tract is under the influence of estradiol, the desquamated cells have deeply pigmented nuclei with a cytoplasm which gives an acidophilic reaction. Few leukocytes are seen. During the midsecretory phase the vaginal smear is predominantly precornified due to the influence of progesterone; the cytoplasm is basophilic with occasional granules. During the late secretory phase (premenstrual), which is two or three days before menstruation, the vaginal smear is composed of tight clusters of precornified cells with polymorphilicnuclear cells present.

Histochemical studies on vaginal tissue demonstrate metabolism and enzymatic activities in different layers of vaginal epithelium. Nucleic acids, proteins, carbohydrates and lipids are distributed through the of enzymes found in the citric acid cycle and fatty acid metabolism. Enzymatic sites for β-glucuronidase, acid and alkaline phosphatase, DPNH-diaphorase, esterase, succinic dehydrogenase and plasminogen activator have been localized with histochemical techniques in the different vaginal cell layers (Fishman and Mitchell 1959; Schmidt and Beller 1978). Glycogen content and glycogen metabolizing enzymes have been studied in female reproductive tissues (Rakoff *et al.* 1944; Feinberg and Cohen 1968; Gregoire *et al.* 1971; Maeyama *et al.* 1977). High glycogen levels are associated with high estradiol levels during the secretory stage of the ovarian cycle in vaginal epithelium (Gregoire *et al.* 1971) while maximum endometrial glycogen levels were evident between the 16th and 23rd days of the cycle (Maeyama *et al.* 1977) thus influenced by progesterone secreted by the corpus luteum. Interestingly, although native glycogen content and the glycogen metabolizing enzymes amylophosphorylase, amylo-1,4-1,6-tranglucosidase and glycogen synthetase were demonstrated in the squamous epithelium of the cervix by Feinberg and Cohen (1968) they reported no alteration in the distribution of either native glycogen or glycogen metabolizing enzymes before or after ovulation.

The wall of the vagina is permeable to a wide variety of compounds and accounts for active transport of certain metabolites from the vagina rather than the blood vessels. Hafez (1977) has reviewed this active transport and absorption mechanism. Absorption from the vagina depends upon size, chemical nature and local pharmacologic action of substances in addition to thickness of the vaginal wall and the presence of specific cytoplasmic receptors. Fats and proteins are absorbed and a number of creams, jellies, supposito-

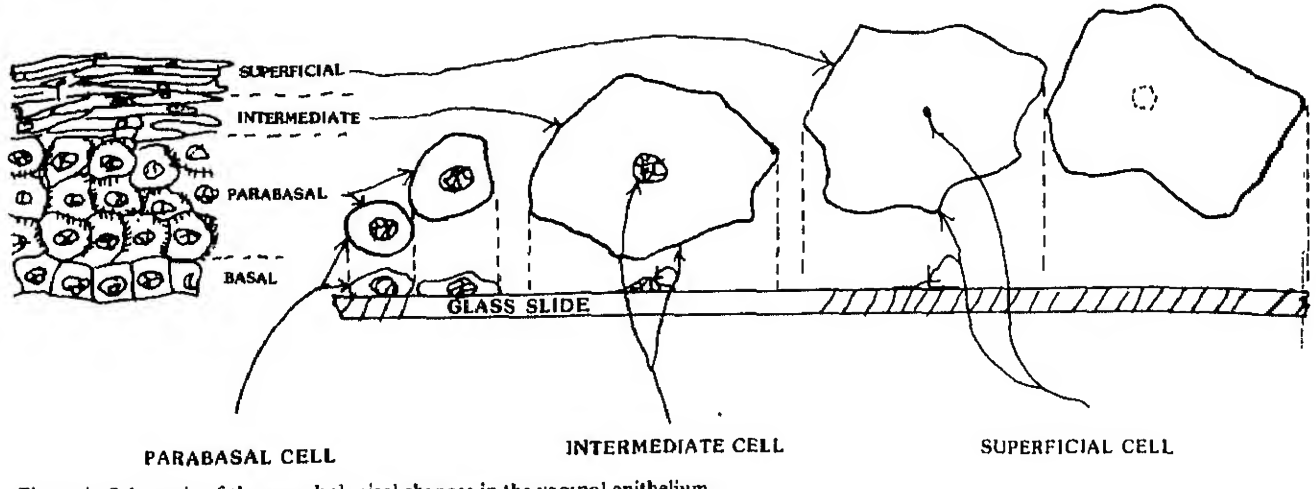

Figure 4. Schematic of the morphological changes in the vaginal epithelium.

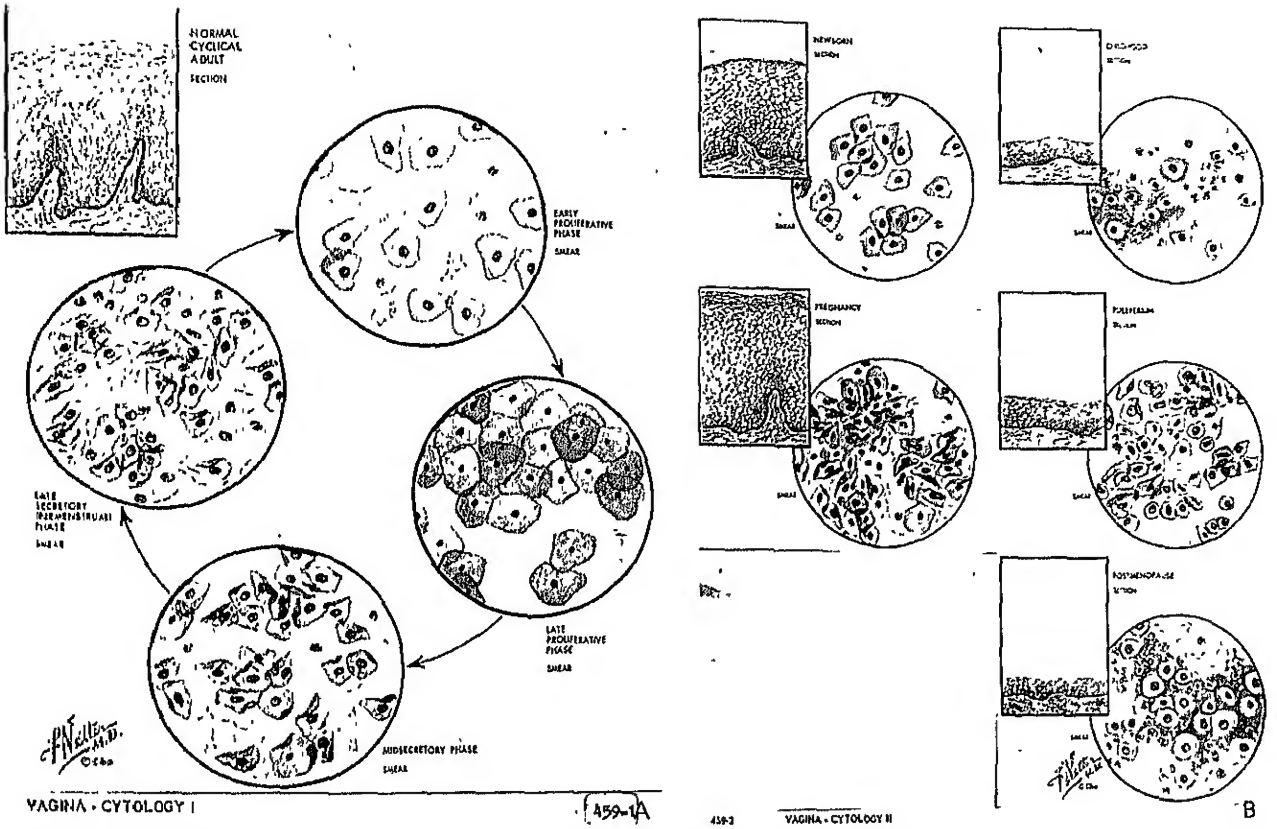

Figure 5. (a) Schematic representation of the changes of the vaginal epithelium during the menstrual cycle. (b) Schematic representation of the vaginal epithelium during the reproductive life of the female from birth through menopause. Under the influence of estrogen in either the late proliferative phase (a) or during pregnancy (b) the epithelium show an acidophilic reaction. When the vagina is not under the influence of estrogen the vaginal epithelium shows a basophilic reaction and stains blue. (Copyright 1965 CIBA Pharmaceutical Company, Division of CIBA-GEIGY Corporation Reprinted with permission from THE CIBA COLLECTION OF MEDICAL ILLUSTRATIONS, illustrated by Frank H. Netter, M.D. All rights reserved)

ries, foams and tablets containing various antibiotics and hormones are introduced into the vagina for therapy or fertility regulation. Following intercourse, most of the seminal plasma is expelled or absorbed through the vaginal walls and is not transported into the uterus. One class of the biochemical components of seminal plasma, prostaglandins, is rapidly absorbed by the vaginal wall and causes oviduct contraction within minutes. Thus the vaginal mucosa is active, responding to ovarian hormones to alter cellular morphology while exhibiting active transport and absorption capabilities for a number of compounds.

Components of the Reproductive Tract

Tables 1 and 2 summarize the components, pH and amount of fluid normally found in the vagina, cervix and oviduct.

There are no active glands in the vaginal epithelium to secrete fluid so most of the fluid volume would be from other sources, primarily from the cervix. There does, however, appear to be some production of fluid in the vagina, presumably through transudation, and this increases during periods of estrogen production or sexual stimulation (Wagner 1979) The active reabsorption of Na^+ from the vaginal fluid back into the interstitial fluid creates a transvaginal electropotential difference thereby providing the mechanism for fluid movement (transudation) through the vaginal epithelium into the vagina (Wagner and Levin 1978) creating a surface film of moisture. Stone and Gamble (1959) were able to recover an average of 0.76 gm of fluid from the vagina with a cotton wad regardless of the stage of the menstrual cycle. There was a peak of 1.0 gm collected at the time of ovulation. The total daily production rate of vaginal fluid has been estimated at 5 gm (Wagner 1979). The cervix, however, does have mucus secreting glandular units that secrete larger volumes of fluid (actually referred to as mucus) around the time of ovulation (Table 2) (Moghissi 1972, 1973). The amount of fluid in the oviduct also varies through the ovarian cycle and reaches an average daily volume of 9.5 ± 3.7 ml (Lippes *et al.* 1981). This fluid serves to nourish spermatozoa and ova and the zygote in the event fertilization has taken place. Although the

Table 1 COMPONENTS OF VAGINAL, CERVICAL AND OVIDUCT FLUID

Vagina	*Cervix*	*Oviduct*
Urine		
Transudate from vaginal mucosa		
Glandular secretions from		
Sebaceus		
Sweat		
Bartholin's		
Skene's		
Cervical secretion	Cervical secretion	
Uterine fluid	Uterine fluid	Uterine fluid
Oviduct fluid	Oviduct fluid	Oviduct fluid
Follicular fluid[a]	Follicular fluid	Follicular fluid
Peritoneal fluid	Peritoneal fluid	Peritoneal fluid
Cellular debris	Cellular debris	Cellular debris

[a]At time of ovulation.

Table 2. COMPARISON OF THE pH, AND QUANTITY OF VAGINAL, CERVICAL AND OVIDUCT FLUID COLLECTED AT DIFFERENT STAGES OF THE MENSTRUAL CYCLE

	Stage of cycle	*Vaginal fluid*	*Cervical fluid*	*Oviduct fluid*
pH		4–5	7–8	7.8–8
Amount[a]	Follicular		.016–.370[b]	0.30–.070[c]
	Mid-cycle	1.0	.050–.570	3.9–9.5
	Luteal		.004–.240	0.6–1.2

The data represents a composite from several sources.
[a]The amount represents that quantity taken at one collection time.
[b]In grams.
[c]In milliliters.

pH of the vagina is acidic (pH4–5), the pH of the cervix (7–8) more closely approximates that of the normal physiological range (around 7.2) and thus spermatozoa are more able to survive.

There are three major classes of flora in the lower genital tract (Brown 1978) that can be categorized as gram positive, gram negative and yeasts. The bacteria normally found in the human vagina are seen in Table 3. The presence of Doderlein's bacillus (Hunter *et al.* 1959) provides a mechanism for converting the glycogen, normally found in the vaginal epithelial cells, to lactic acid and thus serves to maintain the low pH.

There are a large number of chemicals found in the vagina, cervix and oviduct and Table 4 presents some of those that are common, but by no means lists all of the chemicals.

Vaginal fluid consists of a number of inorganic and organic substances and a review of these substances and possible physiological functions has been published (Moghissi 1979). The electrolyte K^+ is 6.6 times greater, while Na^+ and Cl^- are approximately 46 and 61 percent lower, respectively, of plasma levels (Wagner and Levin 1978).

There are a number of volatile organic acids in vaginal fluid and their concentration exhibits consistent changes during the ovarian cycle (Michael *et al.* 1974; Huggins and Preti 1976; Preti and Huggins 1978; Bauman *et al.* 1982). Lactic acid, urea and acetic acid were found in all subjects and the maximum concentration appeared midcycle, at the time of ovulation, with the urea and lactic acid increases occurring from 48 hr before to 24 hr after the LH surge (Huggins and Preti 1976; Preti and Huggins 1978). Some of the other organic acids identified were butyric, propionic, isovaleric and iso-butyric (Huggins and Preti 1976; Bauman *et al.* 1982).

Fourteen amino acids were found in vaginal fluid (Hunter and Nicholas 1959; Gregoire *et al.* 1959). The mean protein content of vaginal fluid is 18 ug/ml (Raffi *et al.* 1977). Raffi *et al.* (1977) identified the proteins in vaginal secretions using immunoelectrophoretic and immunodiffusion analyses. These included albumin, α1-antitrypsin, α2-haptoglobin, α2-macroglobulin, transferrin, β-lipoprotein, γG,K. (Bence Jones) orosomucoid and ceruloplasmin in addition to the immunoglobulins IgG, IgA and IgM. C-reactive protein and fibrin were not identified in vaginal secretions obtained from any of the 29 subjects in this study.

Gaensslen (1983) reviewed the enzymes identified in vaginal fluid. Acid phosphatase (Gomez *et al.* 1975), phosphoglucomutase (Price *et al.* 1976) and lactate dehydrogenase (Divall and Ismail 1983) appear in vaginal fluid. Additional enzymes that appear within the vaginal epithelium and could appear in vaginal fluid include esterase, alkaline phosphatase, β-glucuronidase and DPNH-diaphorase. Recently a vaginal specific peptidase was identified in vaginal fluid (Divall 1984).

There are far more citations describing the inorganic and organic substances in cervical mucus compared to vaginal fluid. The cervix has unique anatomic and histologic structure and secretory functions. Consequently the cervix plays a major role in human reproduction since sperm must be transported from the vagina to the site of fertilization. Cervical mucus has some rather unique properties. These properties are referred to as viscosity, flow elasticity, spinbarkeit, plasticity, tack and ferning (Moghissi 1972, 1973) and change depending upon the stage of the ovarian cycle. These properties therefore are used to assess the stage of the menstrual cycle. The fern pattern, crystallization of the cervical mucus, occurs at the time of peak

Table 3. CLASSIFICATION OF BACTERIA FOUND IN THE HUMAN VAGINA

GRAM-POSITIVE			
Cocci		*Bacilli*	
Aerobic	*Anaerobic*	*Aerobic*	*Anaerobic*
STREPTOCOCCUS STAPHYLOCOCCUS MICROCOCCUS	STREPTOCOCCUS PEPTOSTREPTOCOCCUS PEPTOCOCCUS	LACTOBACILLUS CORYNEBACTERIUM LISTERIA MYCOBACTERIUM	LACTOBACILLUS PROPIONIBACTERIUM CLOSTRIDIUM EUBACTERIUM BIFIDOBACTERIUM ACTINOMYCES
GRAM-NEGATIVE			
NEISSERIA	VEILLONELA ACIDAMINOCOCCUS	ESCHERICHIA PROTEUS KLEBSIELLA SERRATIA CITROBACTER PSEUDOMONAS ACINETOBACTER MYCOPLASMA UREAPLASMA	BACTEROIDES FUSOBACTERIUM

From Brown 1978.

Table 4. COMPARISON OF CHEMICALS FOUND IN FLUID OF THE VAGINA, CERVIX AND OVIDUCT

Vaginal fluid	*Cervical fluid*	*Oviduct fluid*
Lactic acid	Hexose	Lactic acid
Acetic acid	Glucose	Glucose
Urea	Sialic acid	
Butyric acid	Prostaglandins	Phospholipid
Propionic acid		
Albumin	Albumin	Albumin
Transferrin	Transferrin	Transferrin
α1-antitrypsin	α1-antitrypsin	α1-antitrypsin
α2-haptoglobin	α2-haptoglobin	α2-haptoglobin
Acid phosphatase	Alkaline Phosphatase	
Phosphoglucomutase	Esterase	
Peptidase	Aminopeptidase	
Immunoglobulins	Immunoglobulins	Immunoglobulins
IgA	IgA	IgA
IgG	IgG	IgG
IgM	IgM	

estrogen activity, immediately prior to ovulation. An evaluation of these properties is essential so that optimum sperm penetration can be achieved thereby improving the chances of conception (Moghissi and Syner 1970; Blasco *et al.* 1979; Edvinsson *et al.* 1983). As depicted in Table 4 there are a number of different classes of substances in cervical fluid. The properties and biological functions of cervical mucus and interpretation to physiological changes in the female have been reviewed (Schumbacher 1970; Moghissi 1972, 1973). Cervical mucus contains 92 to 95 percent water except at midcycle (at the time of ovulation when estradiol levels are high) when the water content rises to 98 percent thus promoting sperm penetration (Moghissi 1972). The principal inorganic salt is NaCl which increases significantly at midcycle but there are traces of K^+ (Gould and Ansari 1983).

Human cervical mucus includes a number of low molecular weight organic compounds such as hexose, fucose (Iacobelli *et al.* 1972), glucose (Weed and Carrera 1970) and sialic acid (Moghissi and Syner 1976) of which the latter two increase in amount at the time of ovulation. In addition, cervical mucus contains the prostaglandins E_1, E_2, D_2, $F_{1\alpha}$ and $F_{2\alpha}$ (Charbonnel *et al.* 1982).

A number of scientists have studied the proteins in cervical mucus using electrophoretic techniques (Moghissi *et al.* 1960; Moghissi and Neuhaus 1962; Schumbacher *et al.* 1965; Moghissi and Syner 1970; Iacobelli *et al.* 1972). Among those identified were pre-albumin, albumin, α1-antitrypsin, α1-lipoprotein, α1-orosomucoid, transferrin, α2-haptoglobin, α2-lipoprotein, α2-ceruloplasmin, $\beta 1\alpha$-globulin and β1-hemoglobulin binding globulin. The immunoglobulins IgG, IgA and IgM have been identified in cervical mucus (Govers and Girard 1972; Schumbacher *et al.* 1977; Moghissi *et al.* 1980). Enzymes such as amylase, alkaline phosphatase, esterase, aminopeptidase, murami-

dase and components of fibrinolytic enzymes have been identified in cervical mucus, many of which vary in concentration during the ovarian cycle (Skerlavay *et al.* 1968; Smith *et al.* 1970; Moghissi 1972, 1973).

The fluid in the oviduct is formed through the processes of transudation and secretion (Hamner 1973) and serves important physiological roles in promoting capacitaton of the spermatozoa, fertilization of the ova and sustenance of the conceptus. Thus the composition of oviducal fluid changes with the ovarian cycle and during pregnancy. Oviducal fluid has a composition similar to cervical mucus. Some of the proteins reported include albumin, α1-antitrypsin, α2-haptoglobin, transferrin and the immunoglobulins IgA and IgG (Moghissi 1970; Lippes *et al.* 1981).

Forensic Applications

The forensic scientist is asked to identify stains or secretions as being unique to the female reproductive tract during the scientific investigation of sexual assault. Frequently, physical evidence from sexual assault investigations contains mixtures of fluids from the male and female. Identification of menstrual blood on the suspect's clothing would provide useful information in those instances when the female was assaulted during menses. Similarly, the identification of vaginal material in a semen stain would aid in the interpretation of enzyme and blood group substance grouping.

Since vaginal epithelial cells contain high amounts of glycogen (Rakoff *et al.* 1944) forensic methods for identifying menstrual blood (Vagnina 1955; Furuya and Inoue 1966a), blood shed at time of parturition (Furuya and Inoue 1966b), and vaginal debris (Thomas and Van Hecke 1963) have been based upon this glycogen detection. As I discussed in an earlier section, vaginal epithelial cells contain high concentrations of glycogen, particularly when peripheral blood estradiol levels are high during the secretory stage of the ovarian cycle and would stain brown with iodine. However, the glycogen content of vaginal epithelium varies considerably and is absent during childhood and after menopause (Schmidt and Beller 1978) further demonstrating dependency on estradiol. Further, Rothwell and Harvey (1978) demonstrated another limitation to the iodine staining method for identifying vaginal epithelial cells when they were able to iodine stain epithelial cells collected from the urethral opening of the penis. A new peptidase isozyme has been identified in the vagina that apears to be a specific marker for vaginal material (Divall 1984).

A method for identifying menstrual blood by assaying for soluble fibrinogen was reported by Whitehead and Divall in 1973. A subsequent report (Whitehead and Divall 1974) provided an immunoelectrophoretic characterization of this soluble fibrinogen as a fibrin or fibrinogen degradation product. Their findings were supported by Ebert *et al.* (1979) who reported high concentrations of fibrin(ogen) degradation products but could find no evidence for clottable fibrinogen. Gaensslen (1983) has reviewed other identification methods for menstrual blood, including using elevated concentrations of lactate dehydrogenase (LDH) isozymes 4 and 5, but concludes with a discussion on the extensive studies of Stombaugh and Kearney (1978) who stated this method was not satisfactory.

Lastly, I want to discuss the persistence of seminal constituents in the female reproductive tract. The length of time semen can be identified in the vagina following sexual assault could be useful in corroboration of the victim's statements. Of importance is the awareness that the histology and biochemistry of the female reproductive tract is in constant change due to the influence of and variation in levels of ovarian hormones during the menstrual cycle. The lining of the vagina, cervix and oviduct all contribute fluid which would serve to expel semen and dilute semen markers. Seminal components are also absorbed through the vaginal wall. In addition, the orientation of the vagina promotes drainage of seminal plasma, depending upon the position of the female (standing, sitting, etc.).

Davies and Wilson (1974) reported that spermatozoa were usually found in the vagina up to three days after intercourse and occasionally up to six days afterwards. This is in agreement with Willott and Allard (1982) who reported the presence of spermatozoa on casework swabs up to but not exceeding 120 hr. Acid phosphatase of seminal origin, however, is generally detected only within 24 hr of intercourse (Davies and Wilson 1974; Gomez *et al.* 1975). The ability to detect spermatozoa for longer intervals of time in the vagina following intercourse compared to detection of seminal acid phosphatase is due to at least two factors, mobility of the sperm and characteristics of cervical mucus. Sperm will penetrate the cervical canal 90 seconds to 3 minutes after ejaculation into the vagina (Sobrero and MacLeod 1962). Cervical mucus is very tacky during certain stages of the ovarian cycle and swabs of the cervical os have contained sperm 12 days after intercourse (Gaensslen 1983).

SUMMARY

I have reviewed the anatomy and physiology of the female reproductive system with emphasis on the endocrine control of the ovarian cycle, vaginal histology and components of the reproductive tract. Of significance is the discussion of the dynamic and varying influence the ovarian steroids estradiol and proges-

terone exert upon the histologic and biochemical environment of the reproductive tract. I hope this information will be useful and will aid the forensic scientist in the interpretation of physical evidence from sexual assault investigations.

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ANATOMY, PHYSIOLOGY AND DISORDERS OF THE MALE REPRODUCTIVE SYSTEM

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PHYSIOLOGY OF THE MALE REPRODUCTIVE TRACT

This section is designed to provide the forensic scientist with basic information necessary to understand normal male anatomy and reproductive physiology. In a later section we intend to familiarize the reader with the common disorders of the male reproductive system. We recognize that the forensic scientist has to rely upon specimens submitted for analysis from the victim of a sexual assault and occasionally, from the suspect. Together with the narrative of those associated with the sexual assault, the forensic scientist examines the evidence and interprets the data. There are instances, however, when the evidence can be very misleading since there are diseases which render the male incapable of erection (thus penetration would not take place), where the male does not ejaculate, but has erection, and where the male has an ejaculate which contains no sperm. Particularly important to the reader is his understanding that changes in reproductive function, caused by disease or treatment, can lead to a condition in which the male may have sperm in his ejaculate at one point in time but not at another. Thus, although we may provide more information than the average reader cares to assimilate we feel that an extensive review of such subjects as sperm transport, ductal obstruction and drugs is appropriate in a document such as this.

Sexual Differentiation and Maturation

In humans an indifferent gonad capable of developing into either an ovary or a testis can be identified at 3 to 5 weeks of gestation (Figure 1). This primitive gonad is divided into cortex and medulla. The medullary elements dominate if the gonad is to become a testis. Primordial germ cells, which can be identified in the endoderm of the yolk sac during the fourth week, migrate to the gonad and scatter throughout both the cortex and medulla. These primordial germ cells eventually become spermatogonia in the male.

Chromosomal factors determine whether the indifferent gonad develops into an ovary or testis. In general, a testis develops when a Y chromosome is present, and an ovary evolves when no Y chromosome is present.

By the seventh week of gestation, differentiation of the testis becomes apparent. Primary sex cords join in an area that is to become the rete testis and the efferent ducts. Subsequently, the sex cords lengthen and develop a lumen to become seminiferous tubules. These tubules form loops, both ends of which empty into the rete testis. In man, anastomoses between loops, as well as blind-ending pouches, have been demonstrated (Liang 1966), which may have implications in the genesis of intratesticular ductal obstruction.

It is not clear whether the rete testis originates from the primitive gonad or from the mesonephric tubules (primitive kidney). The efferent ducts and possibly the proximal caput epididymis, however, arise from mesonephric tubules. By contrast, the bulk of the epididymis, the vas deferens, and the seminal vesicles arise from the Wolffian or mesonephric duct (Figure 1). Differentiation of the internal genitalia begins in the second month. Mullerian and Wolffian duct primordia coexist in the young embryo. Thereafter, the testis releases a protein substance called Mullerian inhibiting factor (MIF), which is distinct from fetal androgen and causes regression of the Mullerian ducts (Jost 1954; Josso *et al.* 1975). By contrast, testosterone, also secreted by the fetal testis, directs differentiation of the Wolffian ducts into the vas deferens, seminal vesicles, and epididymis. Since these substances act locally, a given individual may develop female ducts on one side, if there is an abnormality of fetal gonadal function (an ovary, streak gonad, or absent gonad), and male ducts on the other (a testis).

The external genitalia in both sexes develop from a common anlage. When fetal testosterone is present,

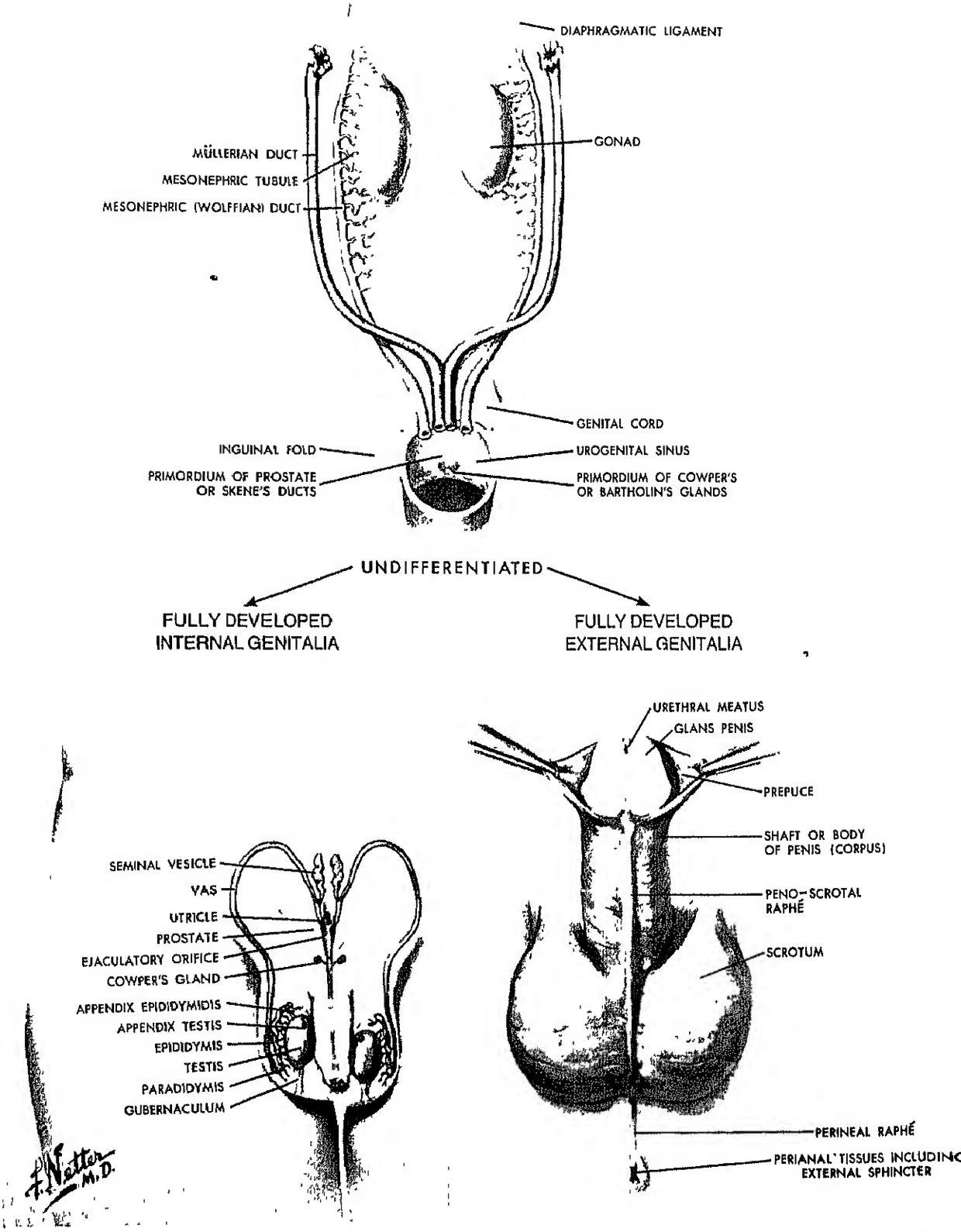

Figure 1. The anatomic structures of the undifferentiated reproductive system and its development into the fully developed male internal and external genitalia. (Copyright 1965, CIBA Pharmaceutical Company, Division of CIBA-GEIGY Corporation. Reprinted with permission from THE CIBA COLLECTION OF MEDICAL ILLUSTRATIONS, illustrated by Frank H. Netter, M.D. All rights reserved)

the genital tubercle, genital folds, and genital swellings give rise to the glans penis, shaft of the penis and scrotum, respectively (Figure 1). Dihydrotestosterone (DHT), a metabolite of testosterone, is the intracellular androgen that effects masculinization of the external genitalia. Thus, to develop normally, those tissues must contain the enzyme 5-α reductase to convert testosterone to DHT.

The testes usually descend into the scrotum during the seventh or eighth month. Approximately 3 percent of full-term infants and 20 percent of premature infants have undescended testes. Seventy-five percent of these undescended testes reach the scrotum by the age of 1 year, at which time the true incidence of cryptorchid testes is 0.9 percent. After birth the testes grow slowly until puberty, when testicular enlargement accelerates rapidly coincident with onset of spermatogenesis.

The mechanisms that affect the initiation of puberty have not been elucidated. Sexual development is preceded by increased secretion of the pituitary gonadotropins (LH and FSH). This results in testosterone secretion, which in turn causes the familiar virilization characteristics of the adult male. The first visible sign of puberty in the male is enlargement of the testis, which occurs approximately at 11 years (Marshall and Tanner 1970). Pubic hair is usually initiated at 13 years and approximately a year later, the penis lengthens. Peak height acceleration occurs at about 14 years. The entire pubertal process extends over 4 years and is usually completed by the age of 15±2 years.

Endocrine Function of the Testis

The availability of chemical techniques for the purification of hormones and radioimmunoassays for the quantification of hormone levels in biological tissues has allowed investigators to gain insight into endocrine function and pituitary regulation of the testis. The release of LH and FSH from the pituitary is regulated by a protein hormone (GnRH) from the hypothalamus of the brain (Schally *et al.* 1971) (Figure 2).

Testosterone synthesis within the testis is mediated by LH (Figure 2). The mechanism by which LH stimulates the Leydig cells to produce androgen is complex. LH binds to receptors on the Leydig cell surface and effects an increase in the activity of the enzyme adenyl cyclase which in turn facilitates testosterone synthesis by an undetermined mechanism. The Leydig cells have the capacity to synthesize steroid hormones from either acetate or choloesterol. The process by which the androgen is released from the Leydig cell into the blood and into the seminiferous epithelium is poorly

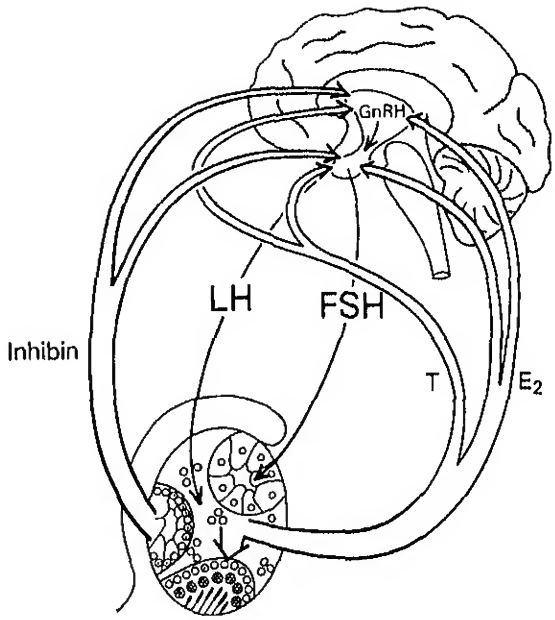

Figure 2. Hypothalamic-pituitary-testicular interrelationships. GnRH (gonadotropin releasing hormone) originates from the hypothalamus and causes LH and FSH release from the pituitary. FSH affects the germinal epithelium and LH acts on the Leydig cells. Testosterone and estradiol exert local effects on the testis and feedback upon both the hypothalamus and pituitary thereby modulating GnRH, LH and FSH secretion. Additionally, inhibin produced by the seminiferous epithelium may modulate GnRH, LH and FSH secretion.

understood. The production of androgen begins to increase in early puberty and reaches adult levels of approximately 7 mg per day after puberty. Testosterone is released from the testis as intermittent bursts because of episodic secretion of GnRH and LH. Other sex steroids are also secreted from the testis, including DHT, 17α-hydroxyprogesterone and estradiol. Most

lates protein synthesis and the production of an androgen-binding protein (ABP), an action also mediated by the enzyme adenyl cyclase and facilitates completion of the spermatogenic process. Although the precise role of FSH is not defined, spermatogenesis requires the presence of FSH and high intratesticular levels of androgen. The presence of specific androgen receptors in the seminiferous tubule supports the view that a high intratesticular concentration of testosterone is necessary for spermatogenesis.

The testis, in turn, regulates gonadotropin release from the pituitary. Classically, feedback regulation of LH secretion has been thought to be directly controlled by testosterone (Figure 2). Recent studies, however, have demonstrated that estradiol also exerts an inhibitory influence on LH secretion (Sherins and Loriaux 1973). Although testosterone and estradiol modulate LH release by different mechanisms (Winters *et al.* 1979), it should be recalled that testosterone serves as the prehormone for estradiol production.

There appears to be specific feedback regulation of FSH secretion by the seminiferous epithelium (Figure 2). A nonsteroidal factor called "inhibin", produced by the seminiferous epithelium, has been suggested on the basis of numerous studies in which FSH concentration has been found to be markedly increased when testicular germ cells are depleted (Van Thiel *et al.* 1971; Baker *et al.* 1976). Evidence is accumulating that inhibin originates in the Sertoli cell and may be a protein. Plasma FSH concentration increases in proportion to germ cell loss within the tubule while LH and testosterone levels generally remain within the normal range (Figure 3).

Sex steroids also modulate FSH secretion under normal and pathological conditions. Recently, the "inhibin" theory of regulation of FSH secretion has been challenged by the demonstration in the rat that testosterone alone can maintain FSH levels within the normal range in the absence of the testis (Sherins *et al.* 1982). Further, a "selective" increase in FSH level can be induced in the absence of the testis when testosterone production is reduced while estradiol production is increased. Studies of men with germinal aplasia and "selective" increase in plasma FSH levels indicate that Leydig cell function is not normal, since testosterone production and plasma free testosterone concentration are both reduced 50 percent (Booth and Loriaux 1983).

ılar function is gradually and progressively uring aging. Histologic changes occasionally

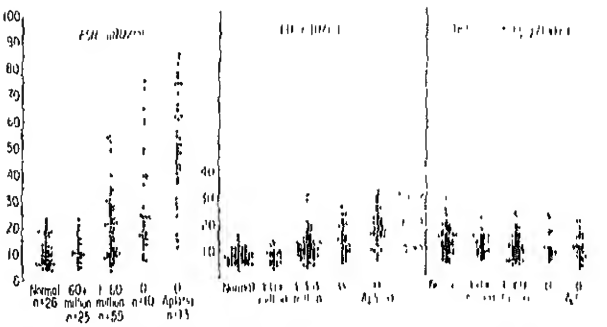

Figure 3. Concentration of serum FSH, LH and testosterone i normal men and men whose total sperm output is <60 million, 1 to 60 million, zero (azoospermia) and zero due to germinal aplasia (From Wachslicht-Rodbard, H. and Sherins, R. J., 1982, Endocrinology of the fertile and infertile male In: Basic Reproductive Medicine, Vol. 2, Reproductive Function in Men Hamilton, D. W. and Naftolin, F. eds., MIT Press, Cambridge, MA)

appear as early as the third decade of life and are increasingly frequent in older men. The most common degenerative changes are thickening of the tubule basement membrane and tunica propria, intratubular fibrosis and reduction in number of germ cells in the seminiferous epithelium. Nevertheless, fertility has been documented in elderly grandfathers (Nieschlag 1982). Mean levels of free testosterone decline in men after age 50, although total circulating testosterone is stable until at least age 70. After 60, there is also an elevation in the plasma levels of estradiol (Stearns *et al.* 1974). These normally occurring changes in senescent testis function become important when assessing the fertility potential of older men.

Functional Role of the Epididymis

In the rat, spermatozoa collected directly by micropuncture from the seminiferous tubules, rete testis or anywhere along the epididymal duct (Figure 4) are not motile in their native fluid. When these sperm are diluted in an appropriate buffer, they become motile to a degree that depends upon their site of collection (Wyker and Howards 1977). Typically, diluted testicular sperm show no motion or slight vibratory tail motion, whereas sperm from the caput epididymis exhibit vibratory activity or disoriented circular swimming. Similar treatment of sperm from the cauda epididymis results in purposeful nondirectional movement. In man, only 12 percent of the caput sperm demonstrate tail movements without sperm progres-

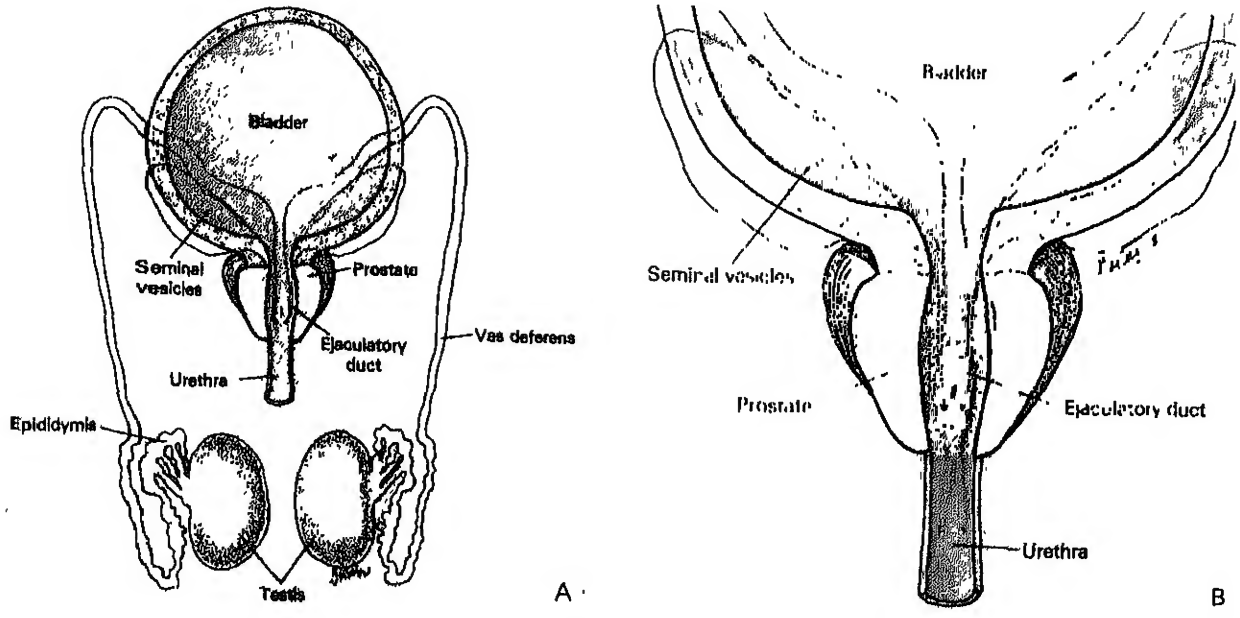

Figure 4. (a) Representation of anatomical relationships among testis, epididymis, vas deferens, accessory glands, ejaculatory ducts and urethra; (b) Representation of anatomical relationships between ejaculatory ducts and numerous independent prostatic duct openings into the urethra.

sion, while 34 percent of the caudal sperm exhibit progressive motility (Mooney *et al.* 1972).

Garbers and associates (1971) demonstrated that dibutyl cyclic 3'5'-adenosine monophosphate (di-cAMP) and cyclic nucleotide phosphodiesterase inhibitors such as caffeine, theophylline, and papaverine stimulate cellular respiration and motility in bovine cauda epididymal and ejaculated sperm. Sperm obtained by micropuncture from the rat caput and cauda epididymides also increase their motility in response to caffeine and di-cAMP (Wyker and Howards 1977). However, these agents do not convert the haphazard motility characteristic of caput sperm to the directed motion typical of cauda and ejaculated sperm. Hoskins *et al.* (1975) have proposed that seminal plasma contains a factor or factors that promote progressive motility in ejaculated spermatozoa.

Caudal sperm are more fertile than caput sperm and this has been confirmed in several species (Orgebin-Crist *et al.* 1975). Testicular sperm are not fertile and spermatozoa held in the proximal caput epididymis acquire an increased ability to fertilize ova, however, they never become as fertile as spermatozoa in the cauda. Therefore, most investigators feel that the changing environment and physiology of the epididymis is critical to the maturation of the spermatozoa.

In addition to alterations in spermatozoa, the luminal fluid changes significantly in the epididymis. A large portion of the fluid secreted from the seminiferous tubules flows into the rete testis and is absorbed in the efferent ductules and proximal epididymis. Inositol, total lipid, and total protein decrease in concentration as the luminal fluid moves through the epididymis (Jesse and Howards 1976). In contrast, potassium concentration rises. Glyceryl phosphorylcholine, sialic acid, carnitine, hypotaurine, and acetylglucosimide are secreted in large quantities into the fluid, but their functions are unknown.

It had been known for some time that the epididymis depends on androgens for maintenance of histologic integrity (Maneely 1959). Subsequently, it was shown that the motility and fertility of epididymal sperm and the secretion of carnitine, glyceryl phosphorylcholine and sialic acid into the fluid are androgen-dependent processes. Vigersky *et al.* (1976) have found androgens and androgen-binding protein in the fluid of the caput epididymis. Although androgen levels are higher in rete testis fluid than are circulating plasma levels, it is not clear whether most of the androgen enters the epididymal lumen from the blood or from the rete testis fluid.

Young (1931) suggested that the epididymis was no more than a holding vessel for maturing spermatozoa. Since that time, there has been a continuing debate as to whether sperm maturation is merely a function of aging or whether the special milieu of the proximal two-thirds of the epididymis plays a significant role in maturation. The evidence cited in the section above on the development of fertility suggests that this capacity depends on a combination of aging and the epididymal environment.

The importance of epididymal maturation of spermatozoa has recently been demonstrated in humans. Bedford and Schoysman (personal communication) have studied the characteristics of spermatozoa ejaculated by patients who had undergone vas to epididymis anastomosis because of ductal obstruction. They found that when the vas deferens was sutured to the proximal 7 millimeters of the epididymis, the ejaculated sperm were immotile, whereas when the anastomosis was distal to the initial 8 millimeters of the epididymis 20 to 80 percent of the ejaculated sperm were motile, and 85 percent became fathers. Silber has shown, however, that sperm which appear in the ejaculate after a vas deferens to caput epididymal anastomosis can markedly improve their motility and fertility potential during several years (Silber 1980).

Transport of Spermatozoa

Mature human spermatozoa are released from Sertoli cells into the lumen of the seminiferous tubule and then traverse approximately 6 meters of duct in the male reproductive tract before they leave the urethral meatus at ejaculation to be deposited in the vagina. This ductal system can be subdivided into several components (Figure 4). From the seminiferous tubule, the spermatozoa travel into the rete testis, a collection chamber for all the seminiferous tubules. The sperm leave the rete testis via the efferent ducts, which in man are composed of 12 to 20 channels, and pass into a single, compact 3.5 to 4.5 meters long convoluted duct known as the ductus epididymis.

The epididymis has been traditionally divided into three regions—caput, corpus, and cauda. Prior to and during ejaculation the spermatozoa move from the cauda epididymis into the vas deferens, which is approximately 38 cm long. The distal portion of the vas deferens is termed the ampulla. The ampulla terminates in the prostatic urethra after it has been joined by the duct of the seminal vesicle, forming the ejaculatory duct (Figure 4). The efferent ducts are lined with cells which project motile cilia into the lumen which may have a role in sperm transport. In contrast, the cilia of the epididymal duct are not true cilia and possess no capacity for purposeful movement.

There is abundant experimental evidence that fluid secreted in the testis flows into the epididymis. Setchell *et al.* (1969) demonstrated that, in the ram, the testis produces up to 40 ml/day (0.5 to 1.5 ml/100 gm/hr) of secretions. If the efferent ducts are ligated, the testis becomes tense and gains weight, and eventually pressure atrophy of the seminiferous tubules occurs (Tuck *et al.* 1970). Since this phenomenon is not seen after ligation of the vas deferens, it is assumed that the epididymis is able to reabsorb the fluid secreted by the testicle. This absorptive capacity prevents permanent atrophy of the seminiferous epithelium after vasectomy in man.

In the human, there is a gradual increase in the thickness of the muscular investment of the efferent ducts, epididymis, and vas deferens. Circularly arranged bundles of small, smooth-muscle-like myoid cells predominate in the efferent ducts and caput epididymis, although some bundles are arranged in a spiral course. In the more distal cauda, smooth muscle cells predominate, forming the three interconnected layers of smooth muscle seen in the vas deferens (the inner and outer longitudinal layers and the intermediate circular layer). The ratio of the thickness of muscle to lumen in the human vas deferens is far greater than in any other structure in man. The reason for such abundant musculature is not known, but it is generally accepted that muscular contractions can aid rapid sperm transport at the time of ejaculation.

The epididymal duct and the efferent ducts contract spontaneously. These contractions are thought to aid in sperm transport even during periods of sexual abstinence, whereas the contractions of the vas deferens and distal epididymis propel the spermatozoa during ejaculation.

Thus, sperm movement into the epididymis may be attributed to several factors: positive fluid pressure from the rete testis, fluid currents established by the beating of cilia along the walls of the efferent ducts and peristaltic contractions of the efferent ducts. Contractions of the seminiferous tubules and contraction of the tunica albuginea have also been implicated in the transport of spermatozoa from the testis. Spontaneous peristaltic-like contractions of the epididymal tubules have been proposed as the sole operative factor in the transport of spermatozoa through the epididymis. Resting hydrostatic pressure gradients may also contribute to epididymal sperm transport.

The elapsed time for epididymal transport of spermatozoa varies from 3 days to 3 weeks in experimental studies. The transport time of spermatozoa, from the time from the testis until storage in the distal cauda, is about 12 days in man. Amann (personal communication) has observed in several species that the transit time of sperm through the caput and the corpus is consistently 3 to 5 days and is independent of ejaculation. Thus, the variation in total epididymal transit time is probably related to differences in the rate of passage through the cauda epididymis, which in turn are due to changes in ejaculatory frequency.

Frequent ejaculations accelerate transit through the cauda epididymis but probably do not affect transport from the caput to the proximal cauda. Amann and Lamequist (1962) have shown that bulls with frequent ejacu-

tions have fewer spermatozoa in the cauda epididymis than those with infrequent ejaculations. Cross and Glover (1958) have shown in the rabbit that stimulation of the hypogastric nerve causes contraction of the vas deferens and cauda epididymis but has no effect on the proximal epididymis. Thus, ejaculated spermatozoa definitely come from the vas deferens and, under circumstances of frequent ejaculation, probably also from the distal epididymis. Pabst (1969) has calculated that the human vas deferens has a capacity of 0.45 ml, which is enough to account for roughly 10 percent of the volume of the normal ejaculate. The bulk of the ejaculated spermatozoa probably comes from the ampullary portion of the vas deferens and is released into the prostatic urethra by contractions of the smooth muscle layers of the organ.

Erection, Emission, and Ejaculation

In order to penetrate the vagina and deposit sperm in it, the penis must be erect. Erection is not a voluntary phenomenon. Men cannot will an erection. Erection following local stimulation (reflexogenic erection) is mediated through the sacral spinal cord, whereas erection following psychic stimulation (psychogenic erection) is dependent on cerebral erotic centers. Psychic stimuli can augment or inhibit reflex erections. Although, classically, erection has been thought of as a simple parasympathetic function, its neurophysiology is complex (Weiss 1972). The afferent nerves for reflex erection run in the pudendal nerves, and the efferent fibers are found in the S2–4 parasympathetic outflow or in the nervi erigentes. The afferent stimuli for psychic erections travel through the thoracolumbar sympathetic outflow and the sacral parasympathetic fibers.

During erection the vascular spaces in the corpora cavernosa and the corpus spongiosum fill with blood (Figure 5). The blood flows to the corpora via branches of the internal pudendal artery—the urethral artery, the artery of the bulb of the penis, the deep artery of the penis. There is vascular communication between the two corpora cavernosa. Conti (1952) has shown that there are physiologic valves located between the arterioles and the vascular spaces in the corpora. When the smooth muscle of these valves contracts, blood is shunted away from the vascular spaces directly into the veins, and the penis becomes flaccid. When the smooth muscle around the valves relaxes, blood fills the vascular spaces, and the penis becomes erect.

Newman and associates (1963) found that in cadavers and living men they could initiate erections by perfusing one corpus cavernosum with 20 to 50 ml of saline per minute. An infusion rate of 12 ml per minute was required to maintain an erection. Neither arterial perfusion in cadavers nor venous occlusion with a blood pressure cuff in living volunteers produced an erection. From these results, they concluded that erection is dependent on blood flow through the corpora cavernosa and is independent of venous obstruction. However, most authorities agree that blood is trapped in the venous spaces by contraction of the ischiocavernosus muscle, which compresses the veins of the corpora against the ischial arch. Beckett and coworkers (1972) have concluded from angiographic and pressure studies in the goat that the arterial inflow to the corpora is also obstructed during erection, thus converting the corpora cavernosa to closed spaces. The fact that Beckett recorded a peak pressure of 7000 mm Hg in the corpora during maximum erection supports this contention. Obviously, psychic, neurologic, vascular, or mechanical problems that interfere with erection can cause infertility.

The ejaculatory process may be divided into two phases, the pre-ejaculatory or emission phase and the ejaculatory phase. During emission, secretions from the periurethral glands, the seminal vesicles, and the prostate are deposited in the posterior urethra (Figure 4). In addition, sperm from the ampulla of the vas deferens, from the vas itself, and probably from the cauda epididymis are propelled by peristalsis into the posterior urethra. The emission process is primarily but not exclusively mediated through the sympathetic nervous system. At the time of ejaculation, the bladder neck (the so-called internal sphincter) closes. This is also thought to be a sympathetic function. Both emission and closure of the bladder neck can be prevented by alpha adrenergic blocking agents (Shishito and Kimura 1973).

The precise neurophysiologic mechanisms of emission and ejaculation are not known. The spinal center for these processes is in the lower thoracic and upper lumbar cord and possibly in the sacral cord. According to Shishito and Kimura (1973), pelvic and hypogastric nerves that stimulate emission are distinct from those that close the bladder neck at the time of ejaculation. There is also evidence in man that emission and bladder neck closure are stimulated from different sympathetic ganglia. Postlumbar sympathectomy patients may have either retrograde ejaculation or failure of emission.

During the final phase of ejaculation, the external urethral sphincter relaxes and the perineal and bulbourethral muscles contract, expelling the ejaculate from the posterior urethra through the urethral meatus. This phase of the ejaculatory process is thought to be triggered by the presence of seminal fluid in the posterior urethra. The fluids secreted by the prostate and seminal vesicles serve as a vehicle of transport for the sperm.

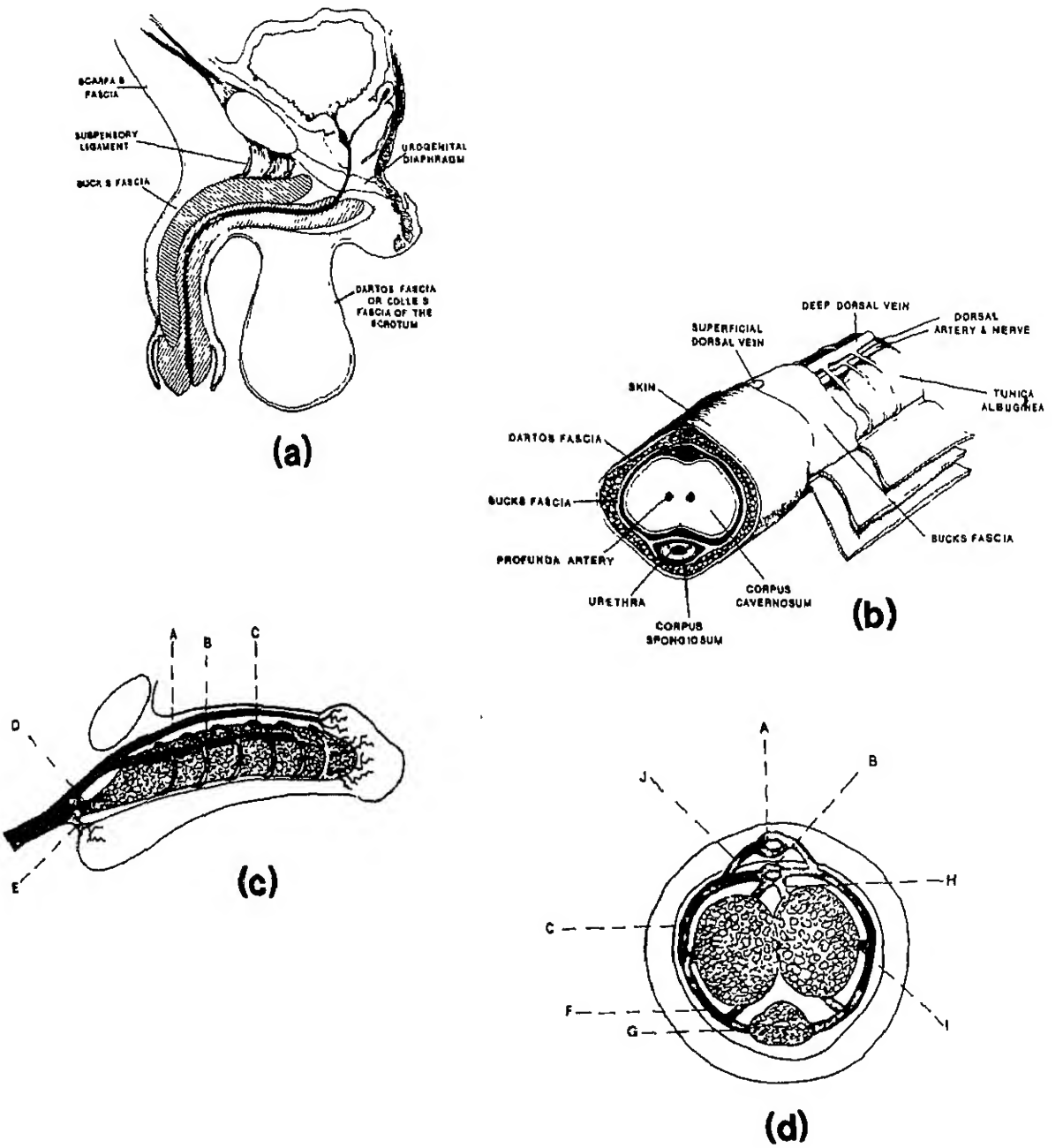

Figure 5. (a) Fascial relationships of the external genitalia. (b) Cross-section of the pendulous portion of the penis illustrating the three cylindri cal masses of tissues; two corpora cavernosa and a single ventral corpus spongiosum. (c & d) Veins of the penis. A superficial dorsal vein; B dee (profunda) dorsal vein, C circumflex vein; D. deep (profunda) vein of the penis; E bulbar vein; F. inferior emissary vein from corpus caverno sum; G. superior emissary vein from corpus spongiosum; H. superior emissary vein from corpus cavernosum; I. lateral emissary vein from corpu cavernosum; J. anastomosis between superficial and deep dorsal veins. (a & b) From, Van Arsdalen, K. N., *et al.*, 1983, Erectil physiology, dysfunction and evaluation. Part I: Physiology of erection. In: 1983 Monographs in Urology, Vol. 4, Stamey, T. A., p. 138 reproduced with permission of Burroughs Wellcome Co. (c & d) From, Newman, H. F. and Northrup, J. D, 1981 Mechanism of huma penile erection: An overview. Urol. 17.401 with permission).

CLASSIFICATION AND ETIOLOGY OF DISORDERS ASSOCIATED WITH THE MALE REPRODUCTIVE SYSTEM

This section presents a classification of disorders of the male based on etiology and patho-physiology. The forensic scientist must understand which diseases and treatments may lead to misinterpretation of sexual assault evidence. Such understanding requires thorough knowledge of the mechanisms which control male reproductive function as well as the diagnostic approach necessary to establish or document the disorder. Table 1 relates the findings on semen analysis to the etiologic classification of male disorders that are now described.

Table 1. CLASSIFICATION OF MALE REPRODUCTIVE DISORDERS BY SEMEN ANALYSIS

A. Absent Ejaculation
- (1) Antihypertensive drugs
- (2) Pelvic, spinal or prostatic surgery
- (3) Vascular occlusion
- (4) Diabetes mellitus
- (5) Psychologic disturbances

B. Azoospermia
- (1) Seminiferous tubular sclerosis
  - (a) Klinefelter's syndrome
  - (b) Chromatin negative Klinefelter's syndrome
- (2) Germinal aplasia
  - (a) Idiopathic
  - (b) X-ray/chemotherapy/toxins
  - (c) Klinefelter's syndrome with mosaicism
  - (d) XYY syndrome
- (3) Maturation arrest
  - (a) Idiopathic
  - (b) XYY syndrome, autosomal translocations
  - (c) Varicocele
- (4) Ductal obstruction
- (5) Exogenous drugs
  - (a) Male hormone
  - (b) Provera
  - (c) Alcohol

C. Oligospermia
- (1) Idiopathic
- (2) Cryptorchidism
- (3) Varicocele
- (4) Drugs
- (5) Systemic illness

Endocrine Disorders

The endocrine causes of male reproductive dysfunction are due to states of gonadotropin deficiency. Hypogonadotropic hypogonadism may be selective, in which case pituitary secretion of other trophic hormones is normal; or it may be associated with multiple trophic hormone deficiencies, as in idiopathic panhypopituitarism or pituitary tumor. The absence of LH and FSH in the prepubertal patient leads to a profound lack of sexual maturation and when it occurs in the adult leads to decreased libido and involution of sexual characteristics. It is essential to have a clear understanding of normal pubertal events. Table 2 lists the major hypogonadotropic syndromes and their relationship to plasma levels of FSH, LH and testosterone.

Table 2. HYPER- AND HYPOGONADOTROPIC SYNDROMES AND THEIR RELATIONSHIP WITH PLASMA FSH, LH AND TESTOSTERONE (T)

Disorders	*Plasma Hormone Concentration*		
	FSH	*LH*	*T*
PRIMARY TESTICULAR FAILURE			
Klinefelter's syndrome	↑↑	↑	↓
Bilateral cryptorchidism	↑↑	↑	↓
Orchitis	↑↑	↑	↓
Germinal aplasia	↑↑	±↑	±↓
Maturation arrest with oligospermia	±↑	n	n
SECONDARY TESTICULAR FAILURE			
Kallman's syndrome	↓	↓	↓
Fertile eunuch syndrome	↓	↓	↓
Panhypopituitarism	↓	↓	↓
Post pubertal panhypopituitarism	↓	↓	↓

Arrow (↑) indicates whether plasma hormone level is increased (↑) or decreased (↓), or normal (n); ± indicates negligible change.

Hypogonadotropic Hypogonadism

Hypogonadotropic hypogonadism can occur as an isolated deficiency of pituitary LH and FSH secretion where the other pituitary trophic hormones are released normally. The disorder may be sporadic, or it may present as a familial defect (Sparks *et al.* 1968). A variety of somatic anomalies have been reported in association with this disorder. The most prominent of these is anosmia (lack of sense of smel
of the olfactory tracts. Other congeni
harelip, cleft palate, mental retarda
epilepsy, cerebellar ataxia, micro
chidism (Sherins and
usually inherited a
(Santen and Paulse
The defect is seen in both sexes.

Delay in sexual maturation is the hallmark of the syndrome. Since prepubertal boys are by definition sexually immature, hypogonadotropic hypogonadism does not become evident until puberty. Although the

alternative diagnosis of constitutionally delayed puberty (late onset of an otherwise normal puberty) can be made only in retrospect, the presence of anosmia or one of the other somatic midline defects may be a clue to the possibility that sexual maturation may not proceed spontaneously. Demonstration of a proper progression of pubertal events is required to differentiate these two states.

Testicular enlargement, which normally precedes peak height growth by 2 years (Marshall and Tanner 1970), does not occur in boys with true hypogonadotropism. Thus, progressive testicular growth indicates that puberty is occurring prior to the appearance of other pubertal features. Testicular biopsy typically shows totally immature seminiferous tubules and absent Leydig cells which are similar to the features of a testis normally seen in a 7-month fetus (Van Wagenen and Simpson 1965). The pubertal testis shows a greater degree of germinal epithelial development.

Unfortunately, measurement of serum LH and FSH, per se, does not distinguish the hypogonadotropic male from a normal prepubertal boy, since serum gonadotropin concentrations in these subjects are within the lower limits of normal in most routine assays (Kulin *et al.* 1967) and the low values are similar to those measured in normal prepubertal children. Although clomiphene administration will increase serum LH and FSH in normal men (Bardin *et al.* 1967), prepubertal boys remain unresponsive to short-term clomiphene administration until puberty, Tanner Stage III (Kulin *et al.* 1971). Thus, clomiphene responsiveness does not distinguish the hypogonadotrophic from the normal boy with delayed puberty.

Recently, luteinizing hormone-releasing hormone (LRF) has become available in most medical centers for diagnostic testing. Most prepubertal children and patients with gonadotropin deficiency release LH and FSH in response to LRF administration (Bell *et al.* 1973). Thus, this test also does not differentiate between normal prepubertal boys and boys with gonadotropin deficiency. The fact that the pituitary responds to LRF indicates that hypogonadotropic hypogonadism is due to an absence of this releasing factor.

Recently, Winters *et al.* (1982) have demonstrated that the increase in prolactin levels which occurs in normal men following chlorpromazine administration is absent in untreated hypogonadotropic subjects and that administration of testosterone restores the prolactin response. Boys with constitutional delay of puberty retain the normal prolactin response to chlorpromazine despite physical features which are indistinguishable from hypogonadotropic males. Thus, it appears that even the very low levels of sex steroid secretion in prepubertal boys is adequate to facilitate the prolactin response from the pituitary following chlorpromazine administration.

Fertile Eunuch Syndrome

Very rarely, a male will present with signs of eunuchoidism but will have large testes (McCullagh *et al.* 1953). Testicular biopsy shows maturation of the germinal epithelium but, Leydig cells are absent. The ejaculate may even contain sperm. These men have been called "fertile eunuchs," and they represent a special subcategory of patients with partial hypogonadotropic hypogonadism.

Because of the testicular enlargement and early signs of virilization, differentiation of this type of patient from a boy who is normally midpubertal may be very difficult. The patient usually fails to progress fully through puberty, and it is the persistent incompletion of sexual maturation that suggests that this is a pathologic state. It is important to note that arrest of pubertal maturation is common with pituitary tumors and this cause of delayed sexual maturation must always be excluded by appropriate radiographic, opthalmologic, and endocrine function testing. However, some men presenting with this syndrome do not have a pituitary tumor and represent only a variant of selective hypogonadotropism (Santen *et al.* 1971).

Since completion of spermatogenesis requires both FSH and high intratesticular levels of testosterone, the fertile eunuch syndrome represents a variant of hypogonadotropic hypogonadism in which there is adequate LH to stimulate high intratesticular levels of androgen, but inadequate testosterone secretion to provide effective virilizing levels of androgen in the peripheral circulation. Accordingly, eunuchoidal proportions develop despite testicular maturation.

Postpubertal Gonadotropin Deficiency

Hypogonadotropism which first appears in a sexually mature male usually results from the development of a pituitary tumor. Not uncommonly, the only pituitary function affected is secretion of gonadotropin. Hence, such an individual may have a loss of libido, decreased potency, and reduced ejaculate volume as a consequence of decreased testicular androgen secretion. It should be emphasized that these symptoms may persist for years before other signs of an expanding pituitary tumor appear, that is, headache, visual field loss, and thyroid or adrenal insufficiency.

Loss of secondary sexual characteristics is a late manifestation of this disease, often requiring 5 to 10 years. By contrast, the testes become small and atrophy early. Testicular biopsy shows sloughing of the germinal epithelium into the lumen of the tubule, eventua

leading to complete loss of spermatogonia. The absence of mature Leydig cells is a most important finding. Laboratory support of the diagnosis of gonadotropin deficiency in the adult male rests primarily with the demonstration of a decreased plasma testosterone level (less than 250 ng/dl), since plasma or urinary gonadotropin concentrations are difficult to distinguish below normal.

Klinefelter's Syndrome

This disorder was described in 1942 as a syndrome of hypogonadal men with small firm testes, gynecomastia, and elevated urinary gonadotropins (Klinefelter *et al.* 1942). This triad of signs results from sclerosis of the seminiferous tubules in association with severe androgen deficiency. With the development of techniques to analyze sex chromatin (Plunkett and Barr 1956) and chromosome complement (Jacobs and Strong 1959), it became apparent that these individuals had an extra X chromosome that was derived from nondisjunction of the chromosomes of the gametes of either parent.

Klinefelter's syndrome occurs in approximately 0.2 percent in newborns (MacLean *et al.* 1964), and in noninstitutionalized men (Paulsen *et al.* 1964). The incidence is significantly higher among men with mental retardation (de la Chapell 1963). Klinefelter's syndrome is the most common cause of hypogonadism.

Approximately 25 percent of men with the clinical triad fail to demonstrate the extra X chromosome (Barr 1966); in these men the disorder has accordingly been referred to as chromatin-negative Klinefelter's syndrome. This nomenclature stems from the lack of understanding of other causes of seminiferous tubular sclerosis as well as from technical difficulties in demonstrating the extra X chromosome (chromosomal mosiacism, Paulsen *et al.* 1968).

The clinical features of the syndrome have been well reviewed (Paulsen *et al.* 1968). Although it has been reported that testis size is smaller in prepubertal boys with Klinefelter's syndrome (Laron and Hochman 1971), a significant decrease in testicular size is usually not evident until pubescence begins. Since some testicular androgen secretion occurs, virilization begins at the appropriate time. However, completion of puberty is delayed. Gynecomastia, eunuchoidism, and impotence are common because of severe androgen deficiency. Occasionally, virilization is adequate but in adult life infertility becomes manifest because of progressive tubular sclerosis and resulting azoospermia. In addition to mental retardation, a wide variety of psychiatric disturbances are common (Becker *et al.* 1966; Theilgaard *et al.* 1971).

Unlike LH, the plasma FSH level is consistently markedly increased in such patients, reflecting profound seminiferous tubular injury (Table 2). Plasma testosterone and LH, however, are within the normal range in 40 percent of patients (Paulsen *et al.* 1968). The apparently "normal" testosterone value results from binding of testosterone to increased levels of the plasma binding protein TeBG, producing a spuriously high total testosterone level. The production rate of testosterone is markedly reduced to 10 percent of normal men (Lipsett *et al.* 1965). Additionally, plasma estradiol (Ruder *et al.* 1974) and urinary estradiol excretion (Gabrilove *et al.* 1970) are minimally increased in the face of marked testicular failure. This imbalance of estrogen to androgen appears to be responsible for the gynecomastia and increased TeBG levels characteristic of the syndrome (Chopra *et al.* 1973).

Although the 47,XXY karyotype is the most common pattern in Klinefelter's syndrome, men with 48,XXYY, 48,XXXY and 49,XXXXY karyotypes have also been reported. The clinical features are generally the same in all, although skeletal abnormalities are more common in association with multiple X chromosomes (Atkins and Connelly 1963).

There are a variety of other chromosomal abnormalities which are associated with testicular dysfunction. Males with mongolism (Down's syndrome) have trisomy of chromosome 21 which is also associated with seminiferous tubular sclerosis (Swerzie *et al.* (1971). Translocations of portions of autosomes (Kjessler 1974) and the XYY karyotype (Baghdassarian *et al.* 1975) can lead to maturation arrest of the germinal epithelium with resulting azoospermia. The XYY karyotype, however, can also be associated with complete germinal aplasia (Santen *et al.* 1970) and seminiferous tubular sclerosis (Balodimos 1966). The clinical and laboratory features, of course, depend on the extent of gonadal injury.

Cryptorchidism

The incidence of infertility in men with cryptorchidism (undescended testis) is controversial. Differences in opinion are often related to patient selection in those studies. Reproductive function in men with abdominal testes is clearly different from that observed in man with testes lying at the inguinal ring. Recognition of the retractile testis, which is caused by a hyperactive cremasteric reflex, is also critical. Scorer and Farrington (1971) suggest that the cryptorchid testis should be categorized as retractile, obstructed, or ectopic.

The reported incidence of unilateral cryptorchidism

varies somewhat depending on the author and the age of the subjects surveyed. Scorer and Farrington (1971) indicate that spontaneous testicular descent continues to occur until age 1 year. The best estimates of the incidence of cryptorchidism are 1 percent at the age of 1 year and 0.8 percent in adult men (Scorer and Farrington 1971).

One scrotal testis should be adequate for fertility. Yet, in individuals with unilateral cryptorchidism the incidence of infertility is higher than expected (Lipshultz 1976). Since descent of the testis into the scrotum requires normal fetal testicular function, some authors have suggested that in the truly cryptorchid individual there is dysgenesis of both testes but maldescent of only one (Charny 1960; Johnston 1965).

There is abundant experimental evidence in rodents that increasing the intratesticular temperature, only a few degrees (42 to 43°C) for as little as 30 minutes, results in damage to primary spermatocytes (Steinberger and Dixon 1959). Similar experiments in men produce oligospermia within 3 weeks that lasts approximately 50 days (MacLeod and Hotchkiss 1941). Thus, retention of a cryptorchid testis in a nonscrotal position may also contribute significantly to its ultimate dysfunction.

Experimental cryptorchidism produces testicular atrophy and dysfunction (Ewing and Schanbacher 1970). Some investigators maintain that unilateral cryptorchidism may also produce decreased testicular mass of the scrotal testis (Hoschoian and Andrada 1975). However, compensatory hypertrophy has been reported in the scrotal testis of some boys with unilateral cryptorchidism where the cryptorchid testis has been entrapped and where the contralateral scrotal testis presumably maintains normal function (Laron and Zilka 1969).

Discrepancies in the fertility potential reported among the various surgical treatment studies probably represent differences in the type of cryptorchidism present in the subjects. Unfortunately, there are no prospective evaluations that relate post-therapy semen quality to anatomic description of the position of the testis noted at the time of surgery. Nevertheless, semen quality is very poor in most men with bilateral cryptorchidism and is poor in the great majority of men with unilateral cryptorchidism despite surgical correction (Lipshultz 1976).

Recent studies demonstrate that despite normal basal levels of testosterone and LH, the response of LH to LRF administration is greater than normal in men with cryptorchidism, suggesting that Leydig cell function is not normal (Lipshultz 1976). Far more striking is the increase in basal FSH concentration and LRF-induced FSH release. These data indicate significant seminiferous tubular dysfunction, a point consistent with characteristically reduced sperm output. Reduced Leydig cell function is more evident in boys with bilateral cryptorchidism, particularly in those with abdominal testes (Rivarola *et al.* 1970).

Varicocele

Varicocele has been recognized for some time as a relatively frequent cause of male infertility. The first reports of improvement in semen quality and pregnancy following varicocelectomy for male infertility appeared in 1929 (Macomber and Sanders). Subsequent literature suggested that there was an important role for varicocelectomy among some infertile men (MacLeod 1965; Dubin and Amelar 1970).

Despite these important clinical correlations, the etiologic relationship of varicocele to infertility remains unclear. A varicocele is an abnormal tortuosity and dilatation of the veins of the pampiniform plexus within the spermatic cord. Ninety percent of varicoceles appear on the left side. This is usually ascribed to the fact that the left testicular vein inserts into the left renal vein. Incompetence of the venous valvular structure may also play an important role. A right-sided varicocele is usually found in association with bilateral varicoceles secondary to venous incompetence of only the left internal spermatic vein.

A variety of mechanisms have been proposed to account for the abnormalities in spermatogenesis associated with varicocele; the most likely is an elevation of intrascrotal temperature due to venous dilatation (Saypol *et al.* 1981). In several studies varicocele was identified in approximately ten percent of the men examined (MacLeod 1969; Johnson *et al.* 1970) and 25 to 50 percent had impaired semen quality. These data, taken together with evidence that many men with varicocele have no difficulty in impregnating their spouses, suggest that the varicocele, per se, may not be a causative factor in infertility but rather a manifestation of a more fundamental testicular disorder. Consistent with this hypothesis is the fact that preoperative varicocele size does not correlate with changes in semen quality or pregnancy rate following varicocelectomy (Dubin and Amelar 1970).

The seminal patterns of infertile men with varicocele have been well described by MacLeod (1965; 1969). Sperm concentration was less than 20 million/ml in 65 percent of subjects, motility was markedly diminished in 90 percent, and the seminal cytology disclosed a prominent "stress pattern" in which tapering, amorphous, and immature cells were abundant. This semi-

nal pattern corresponds closely to the altered testicular histologic appearance reported by Dubin and Hotchkiss (1969); they showed from testicular biopsy that there is prominent germinal cell hypoplasia and premature sloughing of spermatids into the tubular lumen Occasionally subjects may be azoospermic secondary to maturation arrest or germinal aplasia However, the incidence of fertility without surgical therapy is approximately 50 percent among men with varicocele according to MacLeod (1969).

In general, measurements of serum FSH, LH, or sex steroid concentractions provide no insight into this condition. Swerdloff and Walsh (1975) found that men with varicocele have normal Leydig cell function and normal FSH levels in spite of the altered seminiferous tubular function. However, an occasional subject may have an increased serum FSH concentration when germinal depletion is severe.

Ductal Obstruction

The incidence of ductal obstruction among fertile men is approximately 7 percent according to Dubin and Amelar (1971). The causes of ductal obstruction include congenital absence of the ductal system, ductal stricture following infection, vasectomy, and functional obstruction. This diagnostic entity is important to recognize because of its potential reversibility in some instances.

The ductal system may be congenitally absent. Usually there is an associated absence of seminal vesicles, ampulla, vas deferens, and a major portion of the epididymis (Figure 4). Unilateral renal agenesis has also been noted in some affected men. The cause of this developmental anomaly is usually unknown. However, cystic fibrosis, a rare disorder of infancy, is almost always associated with congenital hypoplasia or absence of these excretory ducts (Taussig *et al.* 1972). Because of the absence of seminal vesicles, these patients have, in addition to azoospermia, a low ejaculate volume, semen that does not coagulate at the time of ejaculation, and absence of fructose in the seminal plasma.

Today, vasectomy is the leading cause of infertility secondary to ductal obstruction. It is important to recognize that surgical procedures are available to bypass obstructions of the epididymis and vas deferens; however, pregnancies do not always follow patent reanastomoses.

Finally, certain ejaculatory disturbances can lead to "functional obstruction" of the vas. Any drug or anatomical process that interferes with sympathetic innervation or integrity of the smooth muscle of the bladder neck can alter bladder neck contractility and result in retrograde ejaculation. Interruption of sympathetic innervation can also cause failure of emission. Any man who states that he has an erection and comes to a climax but sees scant semen should be suspected of having retrograde ejaculation. This can be confirmed by demonstrating reduced semen volume and the presence of numerous sperm in a postejaculation urine specimen.

Classically, men with obstruction of the excretory ducts have azoospermia in association with normal testicular size. Testis size, Leydig cell function, and serum FSH and LH levels remain unchanged following obstruction of the ducts (Johnsonbaugh *et al.* 1975).

Lastly, there are congenital deformities of the epididymis and vas. These include absence of the entire vas, absence of the body and tail of the epididymis, obstruction of the distal epididymis with adjoining vas present, obstructions limited to the region between the testis and the epididymis, and cystic changes in the caput epididymis. Lesions at the level of the caput epididymis or rete testis might easily be missed. The findings of McLachlan *et al.* (1975) of the association of sterility, cryptorchidism, cystic changes of the epididymis, and testicular fibrosis in mice prenatally exposed to diethylstilbestrol are very provocative. Studies in men exposed to DES in utero show an increased incidence of epididymal cysts and cryptorchidism but there is no evidence for a direct affect on testicular sperm production (Gil *et al.* 1976).

Drugs

During the past few years, it has been increasingly evident that the seminiferous tubules may be injured by a variety of drugs. The germinal epithelium is a rapidly dividing tissue and, like the bone marrow and gastrointestinal tract, is affected by agents that interfere with DNA synthesis or cell division.

alcoholism characterized by alcoholic hepatitis, fatty liver, and hepatic fibrosis and/or cirrhosis is associated with profound gonadal failure (Van Thiel *et al.* 1974). The hypogonadism of this syndrome is characterized by marked testicular atrophy due to depletion of the germinal cells and peri-tubular fibrosis. Impotence, decreased beard and pubic hair, gynecomastia, and small prostate size secondary to Leydig cell failure are also present. Plasma testosterone levels are generally lower than normal but often remain within the normal range. Because TeBG levels are markedly increased, free testosterone, the active fraction at the tissue receptor sites, is reduced. Thus, total plasma testosterone is a misleading measurement in this syndrome. Plasma FSH and LH levels are variably elevated despite the profound testicular failure because gonadotropin secretion is somewhat blunted due to increased estrogen production. In many respects these patients resemble men with Klinefelter's syndrome.

Recent studies in normal men with good nutrition have demonstrated that after just 5 days of consuming large quantities of alcohol (220 ml/day), there is blunting of the episodic release of testosterone and a fall in mean testosterone levels (Gorden *et al.* 1976). Administration of alcohol for 4 weeks produces further reduction in plasma testosterone levels and a decrease in testosterone secretion. Studies of men during the period of hangover after acute alcohol ingestion likewise demonstrate a reduction in plasma testosterone levels (Ylikahri *et al.* 1974). Thus, it would appear highly likely that alcohol directly alters testicular function.

In a similar fashion, there has been considerable interest in the possible adverse effects of marihuana on testicular function (Maugh 1975). There have been conflicting results as to whether marihuana lowers plasma testosterone levels (Kolodny *et al.* 1974; Mendelson *et al.* 1974). The differences in results may well be due to variations in the definition of "heavy use," the dosage of absorbed cannabis, and the duration of exposure. However, marihuana has been associated with development of gynecomastia (Harmon and Aliapoulios 1972), and Hembree *et al.* (1976) presented data that demonstrate a fall in sperm output 4 weeks after initiation of heavy marihuana exposure. Certainly, more work in this area is required before coming to a definitive conclusion. Nevertheless, cannabis is a potential hazard to testicular function.

Few data are yet available on the effects of most drugs on male reproductive function. But reports of testicular dysfunction and infertility among men exposed to industrial pesticides (Whorton *et al.* 1977; Lipshultz *et al.* 1980) raises serious concern about the effects of many environmental toxins on male reproductive functions.

Yet another category of drug exposure which may alter the seminal features of specimens presented to the forensic scientist is that of androgens. Men born without testes (congenital anorchia), surgical castrates and hypogonadal males, who suffer from primary or secondary testicular failure, can have full virilization, erectile potency and ability to ejaculate restored following the administration of therapeutic doses of testosterone. Under this circumstance semen does not contain sperm because, while the androgen stimulates the seminal vericular and prostatic secretions to development, the testes are either absent or severely dysfunctional. Azoospermic semen specimens can also be induced in formerly sperm positive men who take full doses of androgen regularly since the exogenous testosterone inhibits pituitary gonadotropin secretion which in turn suppresses spermatogenesis, while the exogeneous androgen maintains virilization and secretion of seminal fluids. Since androgens may not be taken by the suspect on a regular basis, sexual assault evidence could vary considerably from what might be expected from an evaluation of a suspect at a different point in time.

Systemic Illness

There is very little information about the effects of systemic illness on testicular function. In addition to the potential toxic side effects of drugs that might be employed to treat an underlying disease, fever, protein catabolism, and the direct influence of the disease process itself on reproductive function may be relevant. Fever has long been known to alter spermatogenesis. Abundant experimental data in rodents and some data in man clearly show that increasing the intratesticular temperature only a few degrees (to 42° C) for as little as 30 minutes results in damage to the primary spermatocytes that is evident within a few hours (Steinberger and Dixon 1959). Similar studies in men by MacLeod and Hotchkiss (1941) show that oligospermia appears within 3 weeks after heat exposure and lasts approximately 50 days.

Changes in seminal cytology following viral and bacterial illnesses are well described by MacLeod (1964). He emphasizes that recovery of the semen may not take place for several weeks or even months following the insult. Not only may the sperm count fall to near azoospermic levels, but the seminal cytology may show a marked decrease in normal oval forms and reciprocal increases in small, tapered, and amorphous cells. Similar changes in the ejaculate can occur after

allergic reactions and emotional disturbances (MacLeod 1964).

Gonadal dysfunction among uremic men is an excellent example of how systemic disease may alter reproductive function. Uremia is well known to be associated with decreased libido, impotence, and infertility (Feldman and Singer 1975; Lin and Fang 1975), and gynecomastia may be prominent (Swerdloff *et al.* 1970). The cause of the metabolic disturbance is unknown Even when patients are undergoing chronic hemodialysis, there is a progressive decline in plasma testosterone and sperm output, which is associated with reciprocal increases in serum LH and FSH levels, respectively (Lin and Fang 1975). Testicular biopsy reveals maturation arrest of the germinal elements and occasionally germinal aplasia. Following renal transplantation, improvement of Leydig cell function and sperm output occurs within 3 months of surgery despite immunosuppressive therapy to prevent rejection.

Sexual Dysfunction

Abnormalities of sexual performance are important to consider when the forensic scientist is unable to confirm sexual assault by identifying semen on physical evidence. The spectrum of disorders may include decreased libido, impotence, premature ejaculation, and failure of intromission. Retrograde ejaculation may occur despite vaginal penetration. The subject is well reviewed by Levine (1976) and Geboes *et al.* (1975).

Decreased libido is often related to states of Leydig cell failure and thus may be a symptom of testicular dysfunction. By contrast some men have normal libido but are impotent, which results in failure to initiate or maintain erection. Depression and anxiety about sexual inadequacies are common psychological factors associated with impotence.

Functional obstruction of the ductal system, vascular insufficiency of the phallus, and alteration of sympathetic innervation can lead to impotence or retrograde ejaculation. Occasionally, a man may be able to maintain an erection but be unable to ejaculate, so-called ejaculatory incompetence (Dubin and Amelar 1972).

Failure of intravaginal ejaculation can also occur. Men with premature ejaculation or severe degrees of hypospadias, chordee, or phimosis may not ejaculate into the vagina, although vaginal penetration may seem to be adequate. Less commonly, the penis may have entered the urethra or anus.

The incidence of sexual dysfunction during rape has been reported as 34 percent (Groth and Burgess 1977). In this study, 170 men convicted of sexual assault consented to interviews about sexual functioning during their assault and their statements were compared to the victim's descriptions of the offense in the police report. Of the 34 percent with sexual dysfunction (58 of the 170 men), 16 percent of the rapists experienced some degree of erectile inadequacy and 18 percent experienced ejaculatory incompetence. Out of those 18 percent with ejaculatory incompetence, 3 percent (5 men) experienced premature ejaculation whereas the remaining 15 percent (26 men) in this category were characterized as having retarded ejaculation. Of the 170 men in this study only 25 percent were characterized as being without sexual dysfunction, although data were not available on 21 percent and dysfunction was not applicable to the remaining 20 percent.

Groth and Burgess (1977) then evaluated hospital records of rape victims where evidence of spermatozoa and/or physical trauma were documented Complete data on the presence or absence of spermatozoa was available on 69 women but laboratory findings confirmed the presence of spermatozoa in only 32 of these cases. Spermatozoa were not identified in the remaining 37 victims, however, there were signs of physical trauma in 27 of these women supporting their contention of assault. Thus the inability to confirm sexual assault by the identification of semen does not preclude sexual assault.

Immunologic Disorders

The possible influence of immune mechanisms on human fertility has been of considerable interest (Mancini 1970; Shulman 1971). The antigenicity of sperm in an experimental setting has been known since Metchnikoff, 1899. Studies of autoimmune infertility have been based either on experiments in which animals have been vaccinated with testicular tissue or on observations of the presence of antibodies to spermatozoa in the sera of some infertile men and women.

Allergic orchitis can be induced experimentally in a variety of species, including the guinea pig, rat, rhesus monkey, and man (Mancini *et al.* 1965). Histologically, the lesion consists of progressive cytolysis of germ cells, leaving behind essentially unaffected Sertoli cells and Leydig cells. The prostate and seminal vesicles appear normal. The histologic lesion appears in 1 to 8 weeks and is reversible after 24 weeks. By immunofluorescence, the antibody has been localized to the acrosome of spermatozoa and spermatids, but probably all germ cells are affected. It is assumed that circulating antibodies are less important because their titers are low and inconsistent, and because no strict correlation exists between their presence and the severity of the gonadal lesion. Thus, cell-medicated toxic-

ity, which is involved in delayed hypersensitivity, may be imported (Brannen *et al.* 1974).

Sperm antibodies in humans have been reported in approximately 3 percent of infertile men and in 20 percent of men with ductal obstruction (Fjallbrant 1968; Phadke and Padukone 1964; Rumke 1968; Rumke and Helliga 1959). Studies of serum from women with unexplained infertility report remarkably high incidences of sperm agglutinins (40 to 80 percent) (Dukes and Franklin 1968; Schwimmer *et al.* 1967). However, serious reservations exist concerning the implications of these studies, since sperm agglutinins were demonstrated in as many as 20 percent of normal women, and sperm agglutinins do not preclude pregnancy (Hanafiah *et al.* 1972). Nevertheless, transvaginal immunization is an interesting concept (Behrman an Otani 1963), and immunoglobulins have been demonstrated in the female reproductive tract (Tourville *et al.* 1970).

Human semen contains numerous antigens. It appears likely that sperm acquire some of these antigens during their transit through the ductal system (Hekman and Rumke 1969), since "coating" antibodies are present in seminal plasma in which no sperm are present. Importantly, only two cases have been reported in which there is documentation of acute allergic reactions to human seminal proteins (Halpern *et al.* 1967; Levine *et al.* 1973). It is not clear why such a syndrome is not more common if the proposed theories of seminal immunization are valid.

SUMMARY

We have provided a review of the anatomy and physiology of the male reproductive system with emphasis on the mechanisms involved in transportation of spermatozoa from the testis, through the epididymis and vas deferens, and then out through the penis at the time of ejaculation. As described the entire process is complex requiring endocrine control by the hypothalamus and pituitary; and neurovascular control influencing some aspects of function, such as erection and ejaculation. We present a number of medical disorders that could interfere with or influence semen delivery or the composition of semen. We have emphasized disorders which are frequently associated with reduced numbers of spermatozoa in the ejaculate or circumstances which could lead to misinterpretation of sexual assault evidence; that is, failure of penetration, penetration without ejaculation or semen without sperm. We hope our presentation enhances understanding and provides clarification of the review of sexual assault evidence by the forensic scientist.

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DISCUSSION

Speakers: *Richard Sherins and Barry Brown*

Poffizen: Can you explain pre-ejaculation and whether such fluid contains sperm? Can you have ejaculation without erection?

Sherins: Many of these terms are poorly defined. With sexual excitement, mental or physical (i.e., visual or other), secretions along the reproductive track begin. In some instances, such secretions could have been there over a long period of time. All men who are sexually competent will have nocturnal erections and

occasionally nocturnal emissions. During a sexual episode, these secretions are formed abundantly and then, depending upon how long before the actual final ejaculation takes place, a significant amount of so-called pre-ejaculation can take place. Emission can take place without ejaculation; such pre-ejaculation fluid would contain sperm.

With regard to whether erection and ejaculation can be disassociated, there's no question that erection can be poor for a variety of reasons (including drugs and neurologic disorders) but not preclude ejaculation. Usually impotence is complete (no erection takes place), so that secretions of the seminal vesicle and prostate don't occur. Not uncommonly, however, we will hear of a patient who claims to have partial erection but can either masturbate or can have a sexual partner arouse to the point where he can produce an ejaculatory response. I don't know whether or not it always contains sperm but I think if the tract is open and partial erection can be achieved, the chances are that it may contain sperm.

Blake: As I listened to your talk, I became very concerned about the survival of my species. I wonder if you have any data on the frequency of aspermia in the general population.

Sherins: Your question is astute. I receive many calls about whether the male fertility potential is changing. The problem is that infertility is not a reportable disorder. As a consequence of this, we do not have any idea about the actual incidence of infertility—and aspermia would be one subfraction of this. If I had to make a guess, I would say that 1 or 2 percent of the male population has infertility due to substantive testicular dysfunction. While there are concerns about environmental toxins, we are going to have to wait until proper epidemiological studies are performed before we can determine whether it's changing.

Brown: A recent publication reported that the age of onset of puberty is declining, and that the number of sperm produced in the ejaculate over the last several years has dropped dramatically. Is this true?

Sherins: Let me caution you again that these reports are not based on solid data. I am unaware of a single broad-based study on sperm production which asks what's happening to sperm production in the general population. One of the things you must remember is that sperm counts have been evaluated only since the 1940's. Since the level of sperm in the ejaculate directly reflects the length of abstinence, a man who contributes a semen specimen after having abstained for 5 days would have relatively high numbers of sperm by comparison to a man who abstained for only 1 day. For the most part, most of the reports of sperm concentration of normal men in the literature fail to constrain the level of abstinence; and there have been no normal men who have been followed prospectively Thus, I would emphasize that there isn't any solid evidence that the fertility potential of the male is changing.

Divall: We also estimate the level of azoospermia at about 2 percent. This is based on the analysis of spots examined in the laboratory where we failed to find spermatozoa. However, all the clinical tests tell us that semen is present.

Sherins: I want you to remember a most important point when you walk out of this room: what you smear from the cloth may be very misleading. Remember, you must ascertain the assailant. I can think of lots of reasons why there may be seminal fluid on the cloth or in the vagina which doesn't contain sperm. I would like you to think about why that could occur. The individual may have retrograde ejaculation, Klinefelter's Syndrome, be taking male hormones, or have had alcohol, kepone, or other toxin exposures that would produce germinal maturation arrest.

Brown: In our experiments, we are finding that we are getting extremely low levels of spermatozoa back from extractions of cloth. We have done studies where we have added a known number of spermatozoa to a piece of cloth (10 million spermatozoa) and then perform the extraction. After counting the spermatozoa, we find that we are recovering only five percent of the spermatozoa off the cloth. In contrast, we recover nearly 100 percent of the soluble components, such as the blood group substances.

Lincoln: Dr. Sherins, you mentioned that there are various causes for azoospermia. I wonder if you can give us any idea whether or not any of these are reversible?

Sherins: This is an important question. Drug and toxin exposure, depending upon which agent and dose

temporary; the individual may have been sterile at the time the therapy took place, but at a later period of time his condition could have returned to normal.

Question: When examining a deposition on cloth, we frequently find many microchemical substances present, but no spermatozoa. Is it possible to have split ejaculates, or would you assume a more homogeneous mixture?

Sherins: Men usually, but not always, have a majority of sperm in the first portion of the ejaculate, and a smaller number, in the terminal part of the ejaculate. The fact is, in the human, differences are relatively small; it is not an all-or-nothing phenomenon. By contrast, in some animals there are more distinct pre-ejaculatory, ejaculatory, and post-ejaculatory components. The stallion has to be bred very carefully. If he gets nervous, as he may with a new handler, he may not be successful in contributing sperm at the proper copulatory moment. In the human, we have no evidence that this kind of careful ejaculatory sequence is important.

Question: In the ejaculatory process, do seminal vesicle secretions and prostatic secretions take place simultaneously or in different periods?

Sherins: I'm not sure. Again, the best answer I can give you is that in some herd animals, these events are discrete and must occur in a very precise fashion. In the human, they are really almost coincidental. It is important for you to remember that some individuals have agenesis of the ejaculatory ducts and can only put out a prostatic secretion at ejaculation. The assailant can be investigated to prove this quite easily.

Schaler: What is the likelihood for multiple ejaculations, and what are the data that describe the differences in semen when multiple ejaculation occurs?

Sherins: There are very few published data, but the few which exist would suggest that sperm reserves are never completely emptied even with several ejaculations per day. The production of sperm refers to how much are being produced by the testicle, not how much is being ejaculated. We know from volunteers who have ejaculated several times a day, that sperm output diminishes so that after about 2 days, there is a nadir of about 15 million sperm per ejaculate. That appears to be approximately what the production of testicular sperm is per day.

Once the sperm are produced, they travel through the epididymis to the vas, where they sit waiting for ejaculation. While abstinence may increase, sperm production continues every day and are stored in an epididymal-vas reservoir of unknown size. Sperm can be lost from this reservoir during a nocturnal emission, or some escape into the bladder and are flushed away with the next urination. With several ejaculations, the numbers of sperm in that reservoir would be depleted to a baseline level of about 15 million. It also takes time to replenish seminal vesicular and prostatic secretions. It is evident to anyone who is sexually active, that with several ejaculations (an ejaculation a day or even two) there would be relatively scant fluid produced. In the normal ejaculate, after 1 or 2 days abstinence, the average semen volume is 3 ml, but after several ejaculations, the volume probably would fall significantly below 1 ml.

Stolorow: In sexual assault, how can you examine evidence and confirm that the vaginal constituents are present?

Brown: The most commonly referred-to technique is the identification of glycogen rich epithelial cells in the extracts of the evidence. As I mentioned, the cytology of the vagina is unique due to these glycogen rich epithelial cells. I would be cautious about this method unless you are confident you can distinguish differences in cell types.

Sherins: Dr. Brown had recently given me an article which indicated that among rapists there is an increase in sexual inadequacy, erectile inadequacy, and premature ejaculation. Are you aware of additional studies reporting sexual dysfunctions among rapists?

Brown: I am familiar with just that one study by Groth and Burgess (New Eng. J. Med., 297:764, 1977) which indicated a prevalence of about 34 percent sexual dysfunction in the population of convicted rapists they studied. They are not able to perform the complete copulatory act. It's well recognized that sexual assault is an act of violence and not a sexual act. There was a recent discussion on this topic at the annual meeting of the American Academy of Forensic Sciences (1983). The consensus of those participating in the discussion was that sexual assault as an act of violence was fairly well accepted.

Blake: The data that we have seen indicates that from randomly selected populations non ejaculation occurs about 25 to 30 percent of the time.

Peterson: What accounts for the opalescence or the turbidity of a semen specimen?

Sherins: The turbidity in semen is due to the presence of sperm cells which are large enough (about 7 microns in length) to scatter light. If a semen specimen contains no sperm, it will have an amber, translucent quality, when held up to the light. Semen with normal numbers of sperm is quite opaque.

Tanton: In casework when we come across an azoospermic condition and we cannot obtain a semen sample from the suspect, what tests can be done with a blood sample that can enlighten us as to the suspect's condition?

Sherins: Because the gonad (and the ovary in the woman as well) functions under control of the hypothalamus and pituitary, and because this control is one of negative feedback regulation, dysfunction of the gonad leads to elevations in gonadotropin concentration. The male is different from the female. In the female, ovarian dysfunction leads to elevation in both gonadotropins. In the male, loss of germinal elements leads to a selective increase in FSH. This is very easily measured in serum by radioimmunoassay. When you are dealing with an azoospermic individual, where the cause is primary gonadal failure (such as the chemotherapy induced azoospermia, or Klinefelter's Syndrome with sclerosis of the tubules), that diagnosis is supported by the finding of elevated serum FSH level. But if you had a suspect with obstruction such as in vasectomy or agenesis of the ducts then the gonadotropin levels would be normal. So you can see that sometimes this test would be useful and other times it would not. A careful medical history and thorough physical examination of the suspect by a trained physician is essential. Additionally, more sophisticated laboratory tests such as a chromosome analysis of blood cells can be helpful in establishing a diagnosis. Further, men taking androgen will have high plasma testosterone levels but gonadotropin concentrations will usually be low due to suppression by the exogenous androgen.

SEMINAL CYTOLOGY

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Semen is a heterogeneous mixture of chemicals and cells, the principal of which are the spermatozoa from the testis. There are a number of classical pieces of literature that eloquently describe the chemical and cellular composition of semen and the biological basis of the male reproductive system and the reader is referred to these documents for a more thorough description of these topics (Bishop 1961; Johnson and Gomes 1977; Johnson *et al.* 1970a,b,c; Mann 1964; Mann and Lutwak-Mann 1981). The chemical components of forensic interest are discussed elsewhere in this publication; I will therefore concentrate on the cellular aspects of semen with the intent of describing these in a manner which will be of value to the forensic scientist in the interpretation of sexual assault evidence.

The Ejaculate

The principal cell in the ejaculate is the spermatozoon (Figure 1). The average ejaculate is 3 ml with a range of 1 to 6 ml (Sherins and Howards 1978). Each ml of semen contains an average of 60 to 100 million spermatozoa in the normal adult male. Seventy-five to ninety percent of the volume of the ejaculate is contributed by the assessory glands: the prostate, seminal vesicles, Cowper's glands and the glands of Littre. The volume of the ejaculate is variable based on a number of factors: time interval from previous ejaculation, metabolic activity of these glands which are male hormone dependent, and causes such as partial ductal obstruction or retrograde ejaculation. The endocrine influence on the prostate and seminal vesicles is principally by the testes. Similarly, the process by which sperm are produced (spermatogenesis) in the testis is under hormonal influence. The adenohypophysis contributes gonadotropins (follicle-stimulating hormone and luteinizing hormone) to stimulate spermatogenesis and the synthesis of androgenic hormones by the Leydig cells of the testis.

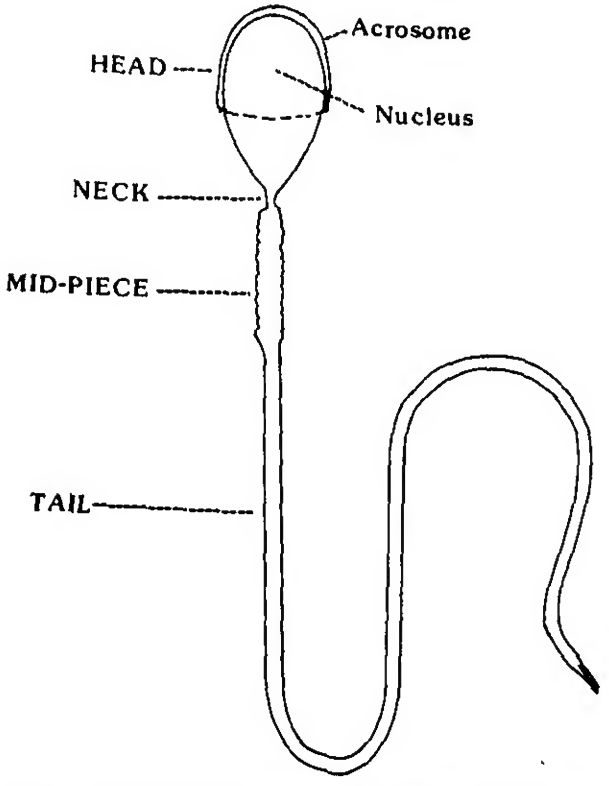

Figure 1. Diagrammatic representation of a mammalian spermatozoon.

Spermatogenesis

The normal testes are made up largely of seminiferous tubules separated by interstitium composed of Leydig cells, blood vessels, lymphatics and connective tissue (Figure 2). The germinal epithelium of the testis is contained within well-defined seminiferous tubules and at the basal layer these cells contribute on a regular basis to spermatogenesis. The process of spermatogenesis has been studied extensively and under normal conditions there is an orderly progression from the primitive spermatogonium type A to the mature spermatozoon (Figure 2). The sequence begins with the dark type Ad spermatogonium, proceeds through the pale type Ap spermatogonium, the leptotene spermatocyte and ends with the spermatozoon (Heller and Clermont 1964). The spermatogonia undergo mitotic division, thus retain their diploid complement of chromosomes, whereas spermatocytes contain haploid germ cell associations in man as described by Heller and Clermont (1963) who defined six stages (I –VI), or cell groups. The cells in these stages progress together into the next cell grouping so that each stage represents a particular degree of maturation. Stages I through VI constitute one cycle. The duration of each cycle in man is 16 days and approximately four cycles of the epithelium are required for an A1 spermatogonium (the more primitive spermatogonium, the stem cell) to mature to a

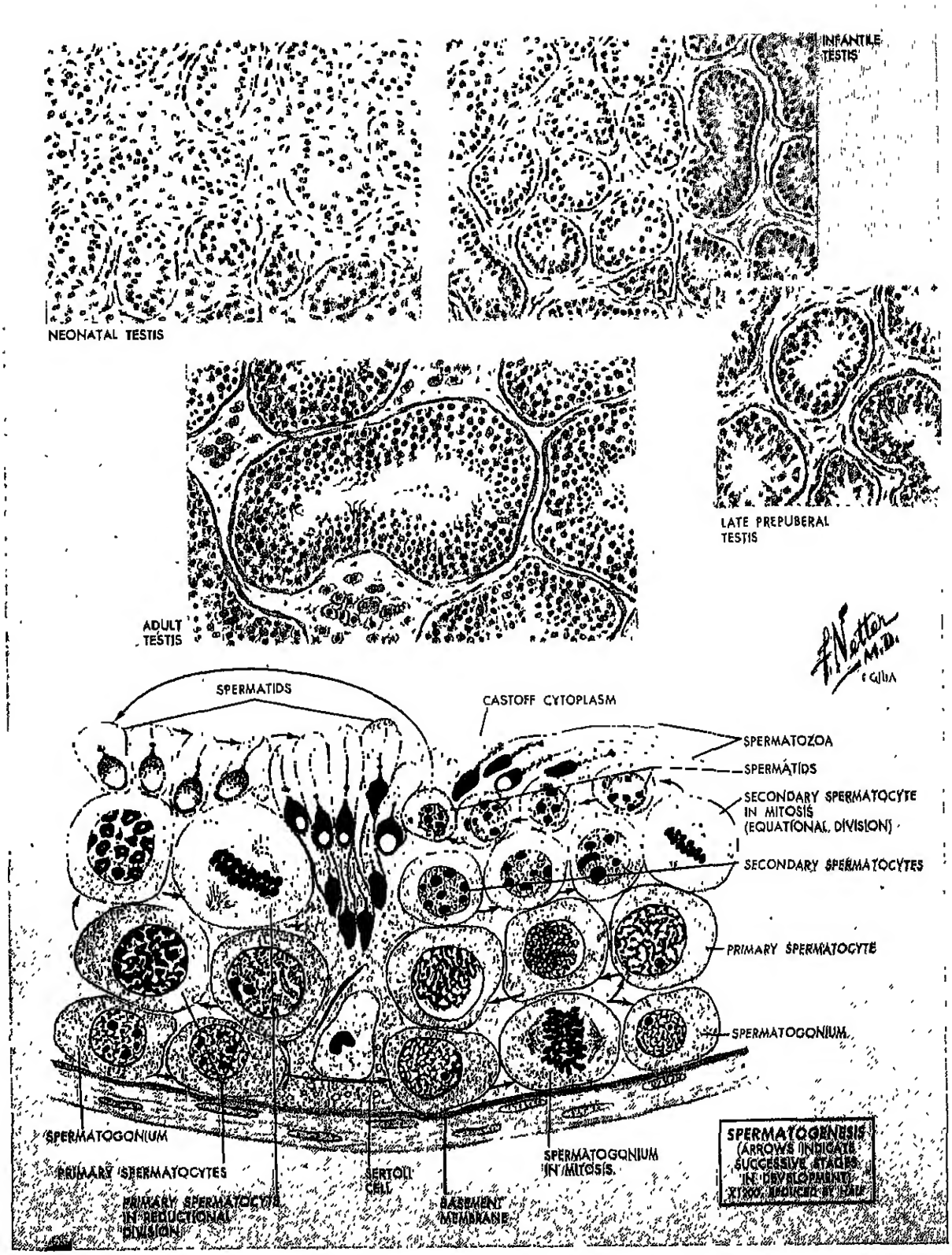

Figure 2. Cross section of the testis showing interstitium composed of Leydig cells, blood vessels, lymphatics and connective tissue, Schematic representation of spermatogenesis beginning with the spermatogonium and developing into mature spermatozoa (Copyright 1965, CIBA Pharmaceutical Company, Division of CIBA GEIGY Corporation. Reprinted with permission from THE CIBA COLLECTION OF MEDICAL ILLUSTRATIONS, illustrated by Frank H. Netter, M.D. All rights reserved).

spermatozoon. Thus it takes approximately 4.6 cycles or 74 days for a mature spermatozoon to develop from a spermatogonium.

Functional Anatomy

Spermatozoa were first observed by the discoverer of the microscope (van Leeuwenhoek 1678). A number of scientists have studied the morphological characteristics of spermatozoa with bright-field, phase and scanning electron microscopes. The mature spermatozoa in man and a number of animal species have a filiform structure owing to the presence of a flagellate appendage. As depicted in the first figure, spermatozoa contain a head, neck, midpiece and tail. The application of a number of techniques and instrumentation has allowed for the detailed study of spermatozoa. Histochemical techniques (Clermont *et al.* 1955), vital stains (van Duijn 1954) and electron microscopy (Fawcett 1958) have been used extensively and now monoclonal antibodies are being used to study both structural and chemical (antigenicity) composition of the spermatozoa (Wolf *et al.* 1982). It should be noted that an estimate of the time since coitus could be determined through careful use of a live-dead stain. Spermatozoa remain viable in the vagina for varying intervals (Breen *et al.* 1972; Shape 1963) and the nigrosin-eosin stain of Dott and Foster (1972) could be used to differentiate the live spermatozoa from the dead spermatozoa.

Spermatozoa have two remarkable functions: motility and fertilizing capabilities. The tail and midpiece contain the mechanisms and enzymatic materials for motility and the sperm head, which incorporates the nucleus, acts as the transferrer of genetic material once the egg is penetrated. The head of the spermatozoa also contains the acrosome, a cap which has the enzymes necessary to penetrate the egg.

The head of the human spermatozoa is oval shaped and appears flattened. The head is about 4.6 μ long, 2.6 μ wide and 1.5 μ thick. The head is packed tightly with chromatin material consisting largely of DNA which contributes to the staining ability when using appropriate stains. Spermatozoa contain one-half of the normal human genetic material and are thus haploid in nature. Since the normal male genotype is XY and since spermatozoa undergo reduction division, half of the spermatozoa will contain the X sex chromatin and the other half will contain the Y sex chromatin. Thus it is the spermatozoa that determine the sex of the offspring. There has been much done in an attempt to accomplish a separation of the X and Y bearing spermatozoa and then to use these separated spermatozoa to influence the sex of the offspring; a recent review by Gledhill (1983) discusses the status.

The tail and midpiece comprise 90 percent of the length of the spermatozoa. There is an axial filament complex that runs down the center of the length of the tail (including the midpiece) which is the motor apparatus of the spermatozoa. This filament is composed of a central pair of microtubular structures surrounded by a ring of nine pairs of microtubules (Figure 3). The ring of nine microtubules is surrounded by a circle of nine noncontractile dense fibers which serve as support structures. These outer dense fibers are formed within a circular fibrous sheath of mitochondria (Fawcett 1975).

MORPHOLOGY

MacLeod (1964) provided an evaluation of seminal cytology that serves as an index of the status of germinal epithelium. Generally, the morphologic picture in a given individual is remarkably constant; abrupt variations in morphologic characteristics reflect an injury to the testis (germinal epithelium) such as an illness (for example, a viral infection), heat, radiation or chemical injury. There is a tremendous amount of heterogeneity in normal human sperm. Some 70 variations in sperm morphology have been described by MacLeod (1965; 1970) and a normal semen specimen may contain approximately 40 percent abnormal sperm forms and may have 2 to 3 percent immature forms. The presence of more than 2 to 3 percent of immature forms suggests injury to the testis such as just described.

There are a number of different stains that are used both to identify spermatozoa and to assess seminal morphology; a description of a large number of these stains is provided in this publication under the section on workshops. The Papanicolau technique described by MacLeod (1964) or as modified by Sherins and

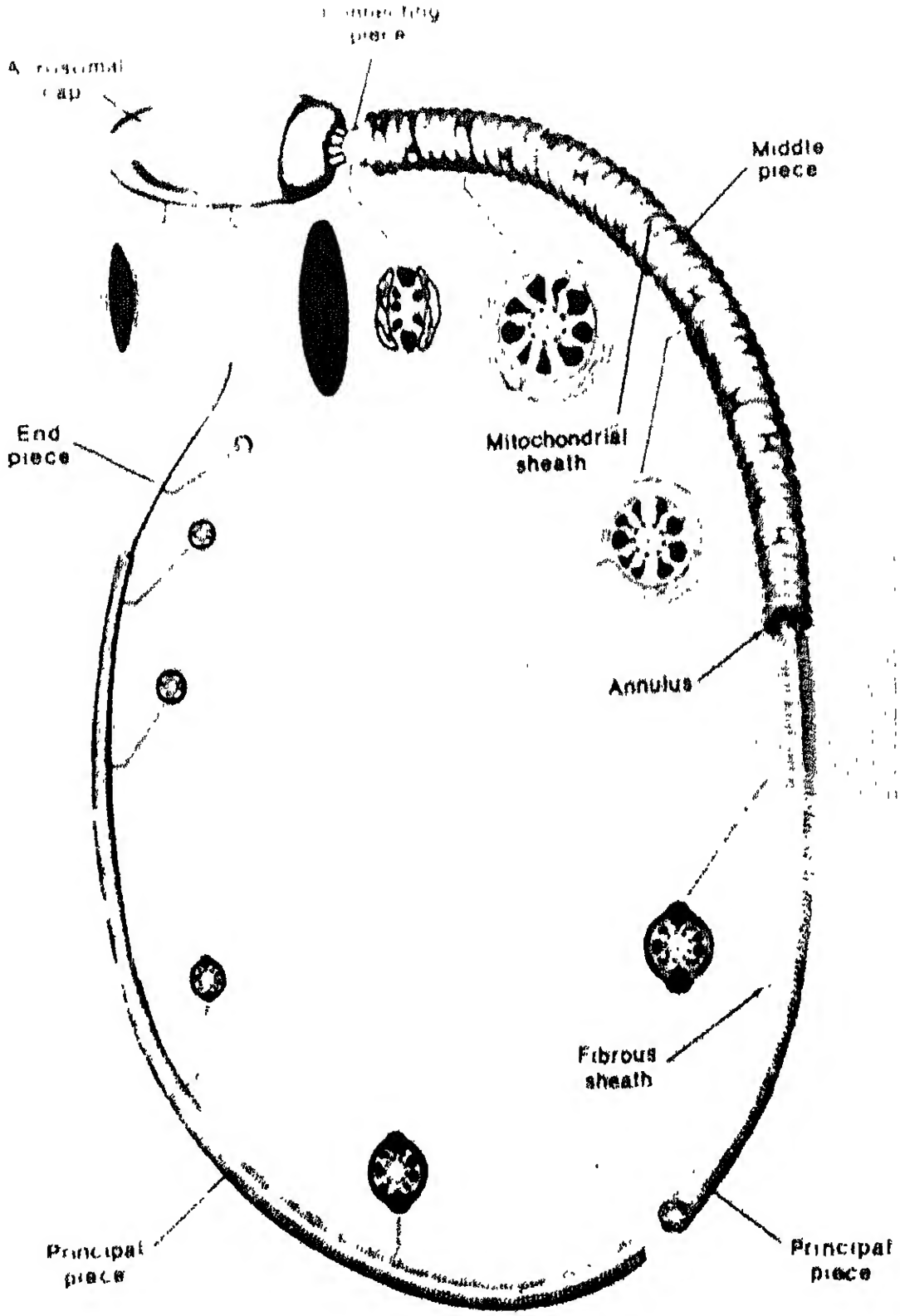

Figure 3. Drawing of the spermatozoa depicting the axial filament complex (From Fawcett, D. 1975, The Mammalian Spermatozoan, Devel. Biol. (44:394).

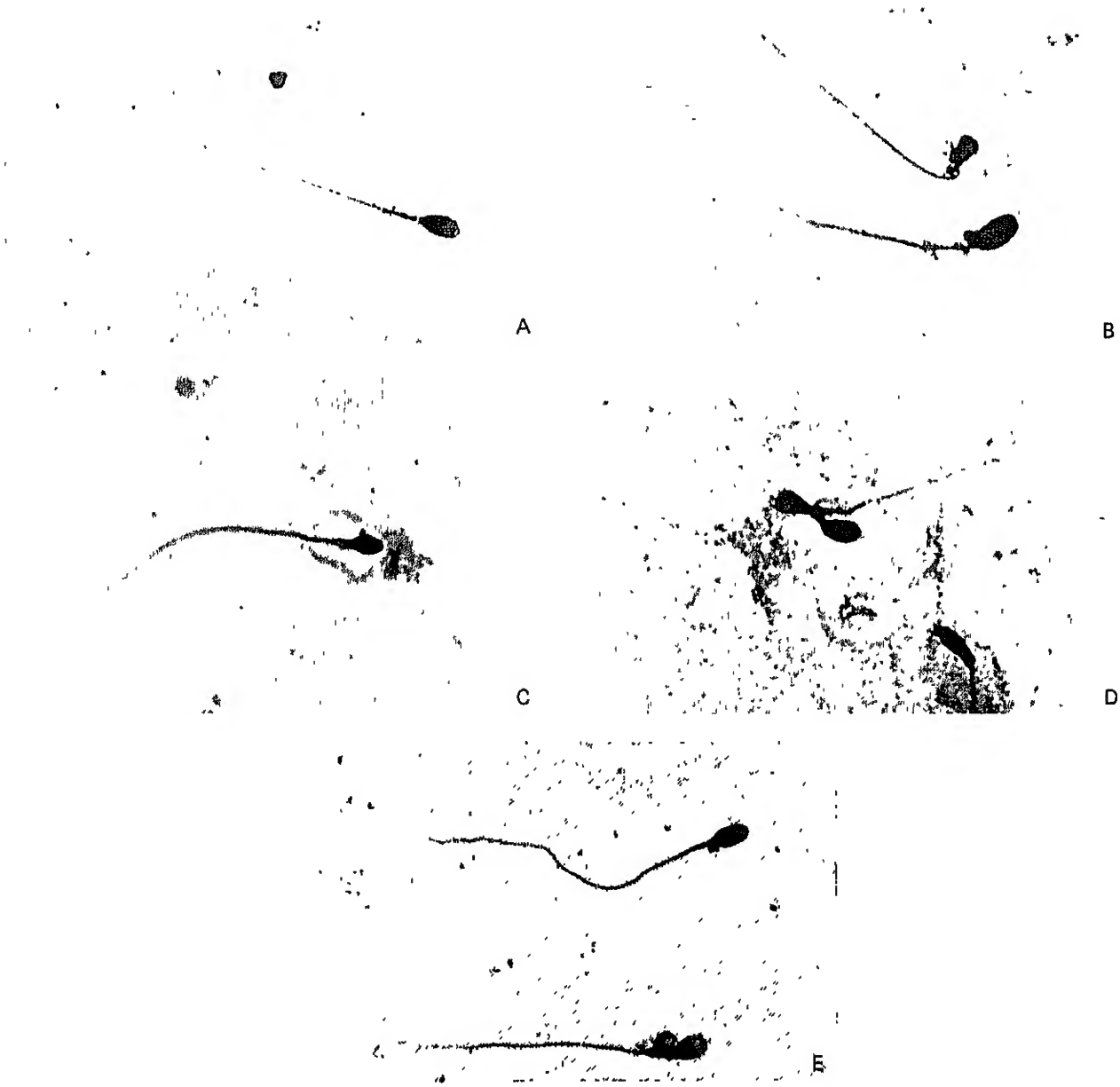

Figure 4. (a) Normal spermatozoa. The sperm in this category generally exhibit a regular oval-shaped head with intact midpiece (approximately 1 μ thick and 7–8 μ long) and an uncoiled tail of at least 45 μ in length. The head is between 3–5 μ and its width must range between 2–3 μ. Sperm with heads which are nearly oval with no gross irregularities are also included in this group provided they fall into the specified size range (e.g., those whose heads are slightly pear shaped). The acrosome, if visible stains pink. The head stains in progressively darker shades of violet from the acrosomal region to the midpiece. The tail stains grey to violet. the head is rounded at the junction to the midpiece in all the oval categories. (b) Large oval head. The large spermatozoa exhibit head shapes as described for the normal category, but the heads are larger. The head is greater than 5 μ in length and greater than 3 μ in width. (c) Small oval head. Small spermatozoa exhibit head shapes as described in the normal oval category, but the heads are smaller. The head is less than 4 μ in length and less than 2.5 μ in width. (d) Duplicate head. Spermatozoa in this category have multiple heads. If a single head sperm possess more than one tail, it is classified under tail defects. The heads may be of various shapes and sizes. The presence of multiple heads takes precedence over any other head classification, e.g., if one or both heads are amorphous, the cell will still be counted as a duplicate head. (e) Cytoplasmic droplet. This category contains sperm with a cytoplasmic droplet (residual body) that is at least one-half the size of the head and is still attached to the head. midpiece or upper tail region. The tail is normal length and shape. (From Belsey *et al.* 1980, Laboratory Manual for the Examination of Human Semen and Semen-Cervical Mucus Interaction, Press Concern, Singapore. The author appreciates the cooperation of Dr. C. Alvin Paulsen for furnishing the above photographs. Credit for these photographs is given to: NIH Grant P50 HD 12629, C. Alvin Paulsen, Professor of Medicine, University of Washington School of Medicine, Seattle, WA 98195 and The Special Programme of Research, Development and Research Training in Human Reproduction, World Health Organization, 1211 Geneva 27, Switzerland).

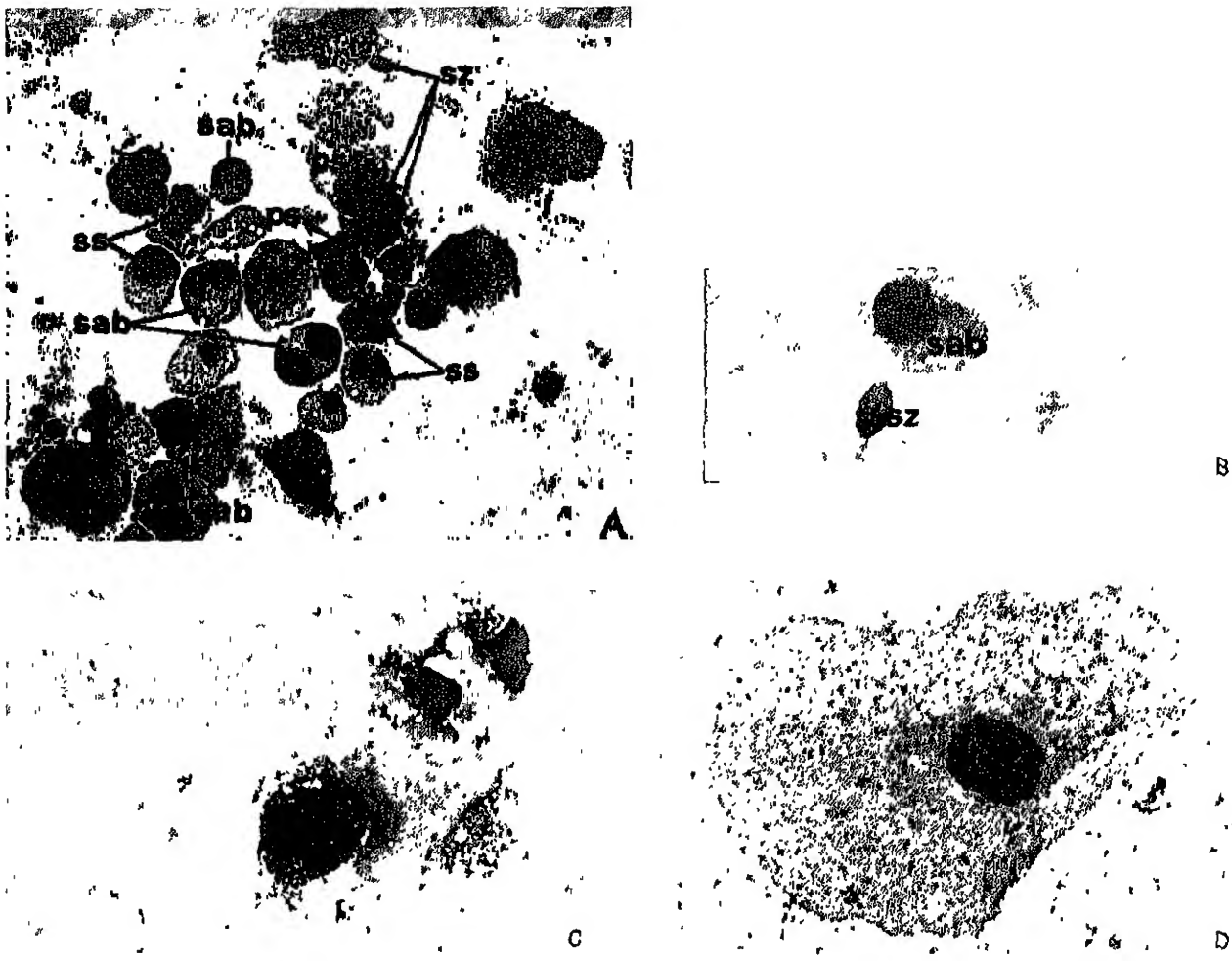

Figure 5. (a) Centrifuged seminal specimen containing a large number of immature germinal cells some of which are undergoing degeneration: ps—represents primary spermatocytes which are characterized by a basophilic cytoplasm and a large round nucleus; the chromatin pattern is sometimes irregular and coarse depending upon the stage of division; psl—represents a primary spermatocyte undergoing division; ss—represents secondary spermatocytes. In this, cells which are smaller than the primary spermatocytes the chromatin pattern is still clearly distinguishable in the nuceli. The cytoplasm is basophilic; sab—represents Sab spermatids. Several of these cells are bi-nucleated indicating an incomplete division of the secondary spermatocytes. In the Papanicolaou stain these bi-nuclear germinal cells often show an eosinophilic sytoplasm which is a sign of degeneration. In the well preserved Sab spermatids the cytoplasm is basic. sz—immature spermatozoa with the tail more or less developed. (b) A spermatozoon and a Sab spermatid. The spermatid has still a considerable amount of basophilic cytoplasm which gradually disappears during the maturation process. The chromatin pattern is partly diffuse due to the beginning of condensation. The size of the nucleus is smaller than that of the secondary spermatocyte but still larger than the Sed spermatid (1300X). (c) Polymorphonuclear leucocyte; (Nuclear diameter 8 –12 μ). These cells have purple to violet nuclei and pale blue cytoplasm. The peroxidase granules, if still active, stain dark purple to black. This effect depends upon the age and condition of the cell and upon the freshness of the smear at the time of staining. Older polymorphonuclear leucocytes and old smears may not exhibit this granular staining. Very young or atypical cells often do not exhibit chromatic bridges or peroxidase granules. The nuceli, however, are usually less than 4 μ in diameter and widely spaced, aiding in their differentiation from Sab spermatids. (d) Epithelial cell: No unusual characteristics. The significance of numerous epithelial cells present in the ejaculate have not been clearly defined. (From Belsey *et al.*, 1980, Laboratory Manual for the Examination of Human Semen and Semen-Cervical Mucus Interaction, Press Concern Singapore. The author appreciates the cooperation of Dr. C. Alvin Paulsen for furnishing the above photographs. Credit for these photographs is given to: NIH Grant P50 HD 12629, C. Alvin Paulsen, Professor of Medicine, University of Washington School of Medicine, Seattle, WA 98195 and The Special Programme of Research, Development and Research Training in Human Reproduction, World Health Organization, 1211 Geneva 27, Switzerland.

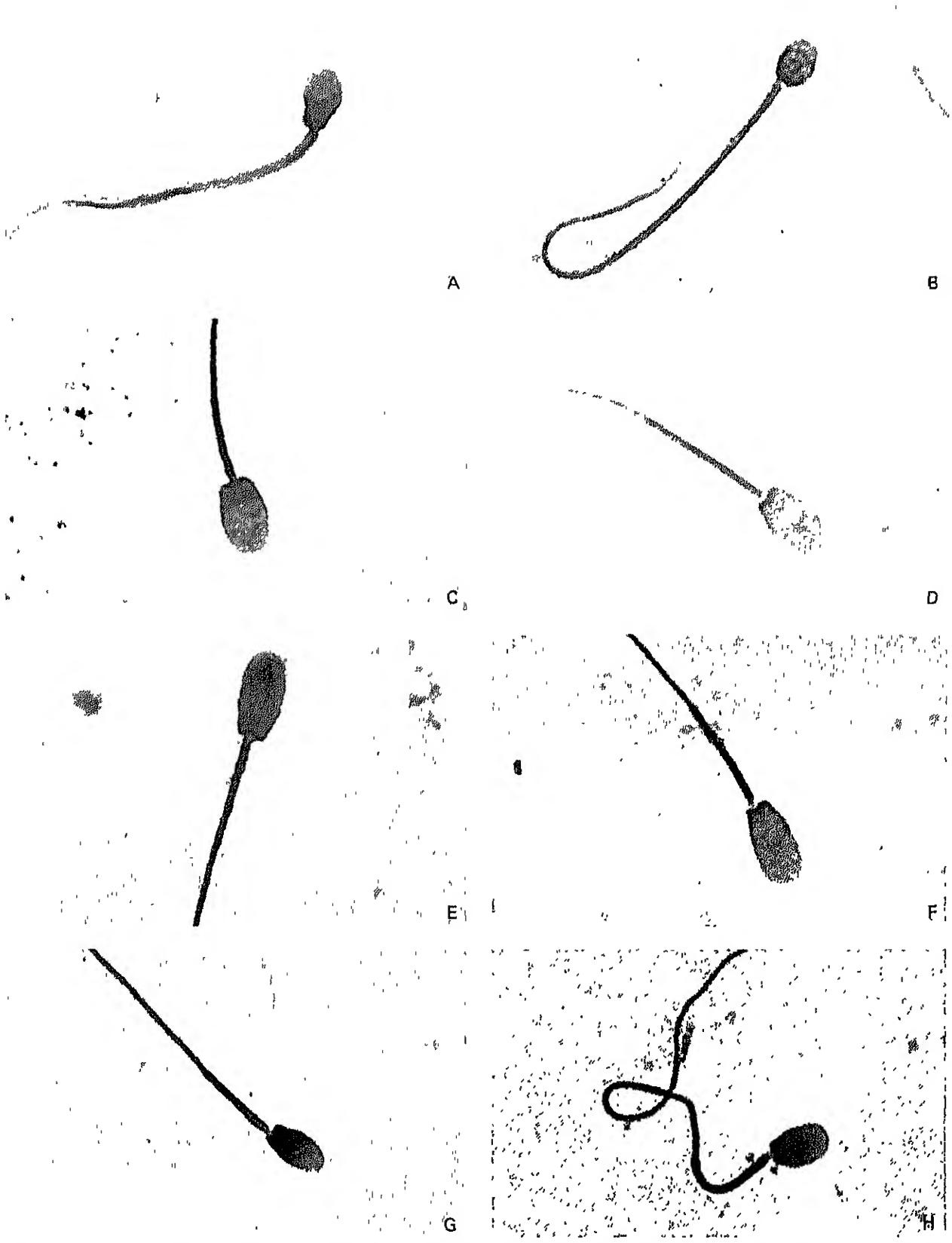

Figure 6. Photomicrographs of spermatozoa illustrating the diversity of form among different species, (a) human, (b) gibbon, (c) domestic rabbit, (d) ram, (e) boar, (f) bull, (g) horse and (h) dog. Magnification 1100X.

hese are primary spermatocytes, secondary spermatocytes and spermatids, some of which are undergoing egeneration. Figure 5b illustrates a spermatozoon nd a spermatid. The spermatid has a considerable mount of cytoplasm which gradually disappears during the maturation process.

In addition to varying forms of spermatozoa there are also leukocytes (Figure 5c) and epithelial cells (Figure 5d) that appear in seminal fluid.

Thus in the normal human male ejaculate, although the majority of cell types are the mature spermatozoa, there are a number of immature spermatogenic cells as well as epithelial cells and leukocytes.

Comparison of Human Spermatozoa with Other Animal Spermatozoa

The total length of a human spermatozoon is about 50 to 60 μ, about the same size as the rabbit, ram, boar, bull and other primate spermatozoa. The shape of the head varies with the species of animal, however. Like the human, the shapes of the head of rabbit, ram, boar and bull spermatozoa are ovoid (Figure 6) and would be difficult to distinguish under the best of circumstances. A comparison of human and baboon spermatozoa is presented in Figure 7 and although there are differences in the shape of the head, these differences are subtle Thus, when confronted with dried spermatozoa where

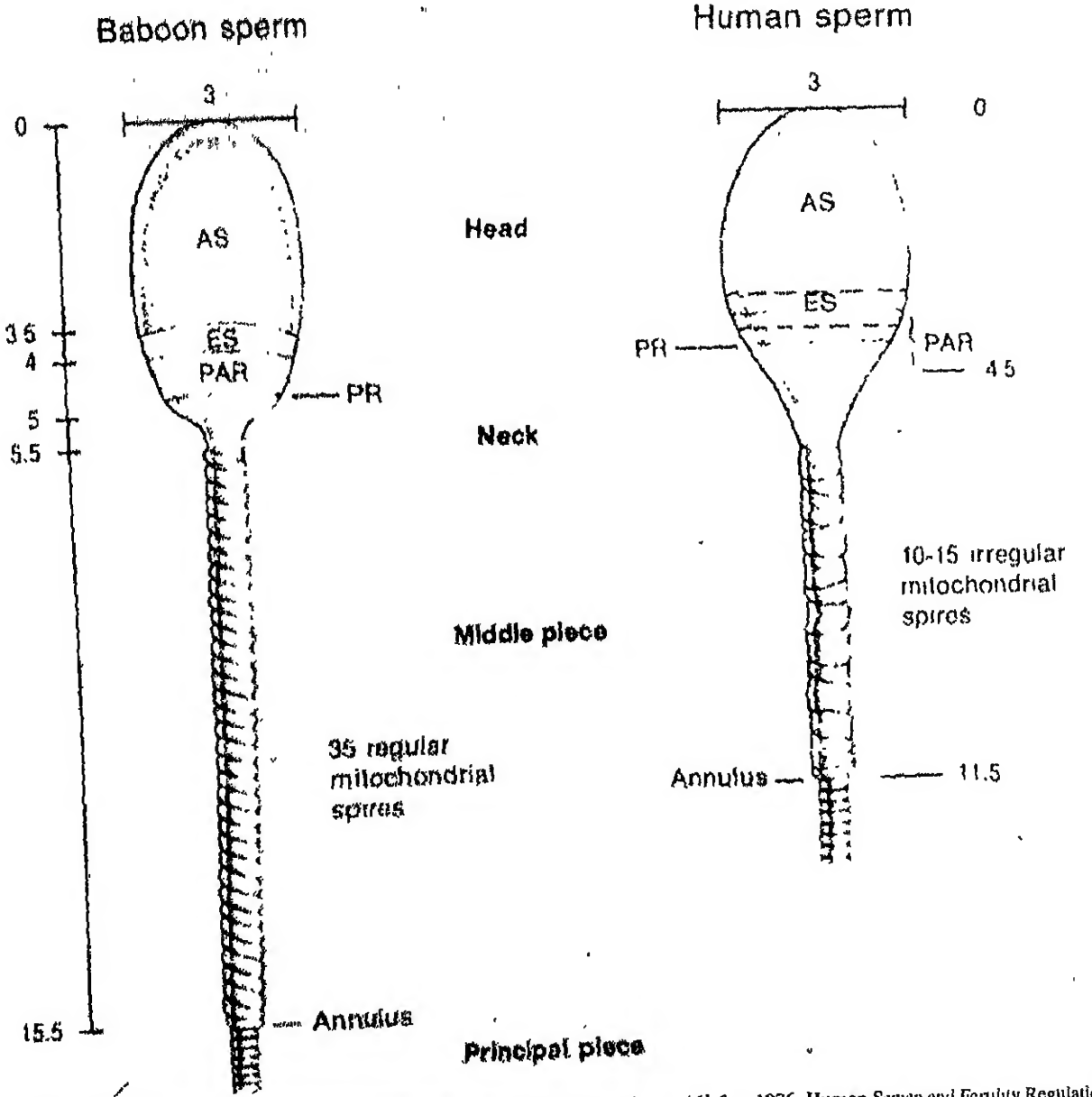

Figure 7. Schematic representation of the human and baboon spermatozoa. (From Flechon and Hafez, 1976, Human Semen and Fertility Regulation, C. V. Mosby, p. 80).

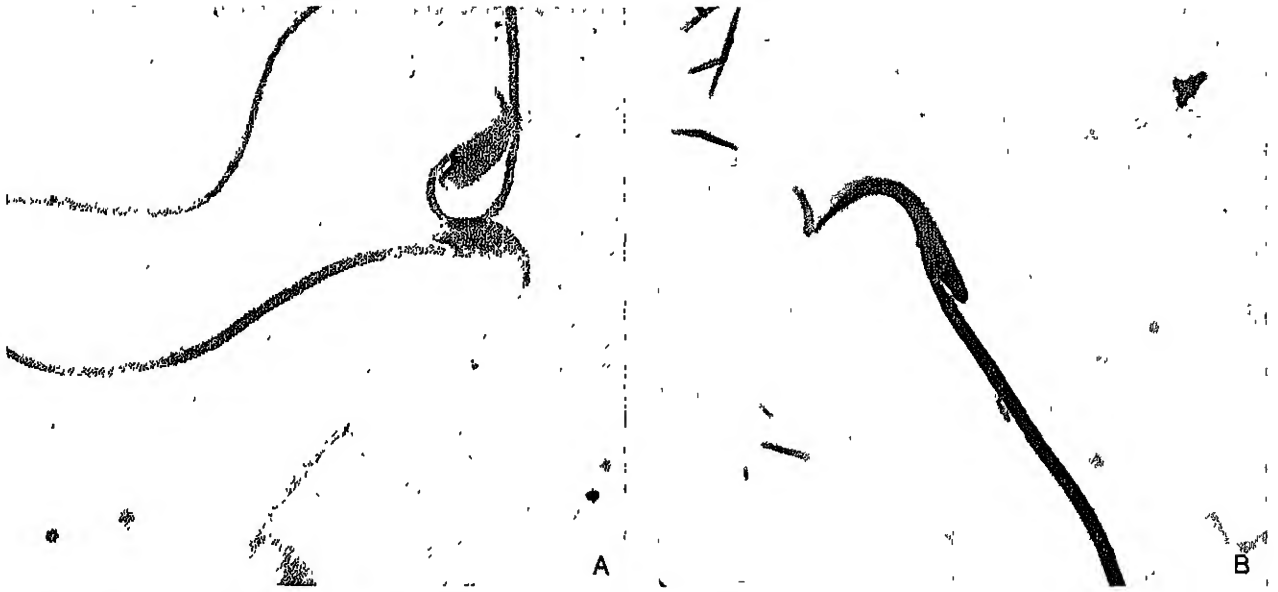

Figure 8. Photomicrographs of spermatozoa of the (a) mouse and (b) rat. Magnification 1100X

one could expect the shape of the head to be somewhat altered, one should note with caution attempts to identify spermatozoa from one of these species using shape of the sperm head as the sole criterion for distinguishing the species. In point of fact, if one had a large number of spermatozoa and upon examination of cells they were found to be similar in size and shape (homogenous mature spermatozoa as the only cell type in the ejaculate), one should become suspicious since, as just mentioned, in the normal human ejaculate only 60 percent of the cells would be mature spermatozoa. In contrast, spermatozoa of the mouse and rat are more easily identifiable due to the distinct hook of the sperm head (Figure 8).

Karyotyping Human Spermatozoa

There are new techniques being developed for application to basic biomedical research that could have application to forensic science. One of these is karyotyping of human spermatozoa and thus providing new methods of identifying an individual. Martin *et al.* (1982) reported a method for direct chromosomal analysis of human spermatozoa by performing *in vitro* fertilization of hamster eggs with human spermatozoa. Following fertilization the haploid complement of the human chromosome could be analyzed. This technique is currently dependent upon viable spermatozoa, thus application of this technique would be limited to those few instances where suitable specimens were recovered in a reasonable time interval following assault.

SUMMARY

In conclusion, the cytology of the human spermatozoa is distinct and lends itself to identification through a number of techniques. In the normal ejaculate there are hundreds of millions of mature spermatozoa in addition to varying forms of sperm cells. Thus the ejaculate is heterogeneous and depicts the range of cell types associated with spermatogenesis.

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DISCUSSION

Question: What percentage of the population have immature sperm cells and abnormal morphologies?

Chang: Dr. Sherins is probably the expert in this area. He has done very elegant studies on the longitudinal analysis of seminal plasma. It's very difficult to answer that question, because it is very difficult to define what a normal population is. But what we can say is that in a "normal ejaculate," 60 percent of the sperm have "normal morphologies;" that means that 40 percent have abnormal morphologies. If you look at a fertile population, on the average, 40 percent of the cells ejaculated have abnormal morphologies. Now with respect to immature sperm cells, I can't say. Maybe Dr. Sherins knows.

Sherins: There are about 1 or 2 percent spermatids in most ejaculates of men who have large numbers of sperm. Occasionally, if you look at sequential samples of one individual, you won't see any spermatids at all; but if you just sit at the microscope and look carefully, there are a large number of abnormal forms in the ejaculate of all men who produce sperm. So its ubiquitous in 100 percent of the human population. We don't see a homogeneous ejaculate at all. The distribution of the abnormal types would favor mostly amorphous, a few are tapered, and about 0.5 to 2 percent are spermatids and, not uncommonly, leucocytes.

Chang: In addition, the results that you get in any seminal analysis may differ depending on which laboratory you send the results. This reflects the experience in the training of the technicians doing the analysis. In the case of varicocele that Dr. Sherins described the prevalence of immature cells increases dramatically. So, instead of having 60 percent normal forms, you may easily have 80 percent abnormal forms. In cases of pathology, the abnormal morphologies of the mature sperm cells and the presence of immature sperm cells can be greatly increased.

Question: Is there a seminal fingerprint?

Chang: There is always the possibility of finding a person with a characteristic seminal cytology. However, if you were to look at the entire population

general and try to pick out specific characteristics for this type of person or that type of person, it would be very, very difficult to document. Sperm cytology changes, and it changes from person to person. Within a single person you can have variability as well. So the inference of a seminal fingerprint, in my estimation, would be only marginally viable. I would think there is enough variation in the normal population that these fingerprints would not be consistent at all, and I would be very dubious about finding a strict seminal cytology for any given person over a long period of time.

Divall: Are the epithelial cells from the vagina in any way specific? Can they be confused or differentiated, in any other aspect, from epithelial cells from the male reproductive tract?

Chang: I would have to answer that by saying if you took any epithelial cell from the male reproductive tract and subjected it to aging processes and to an environment, such as in the female reproductive tract or the environment of the clothing, these cells begin to degenerate. So, you are not looking at a normal cell anymore. The question is: How does a sperm look as it degenerates? The answer is: the nucleus does become pycnotic and in the normal senescent process of most cells, the cytoplasm becomes acidophilic. Now in terms of being able to say that if you see a pycnotic nucleus, it is only from a female—that is not correct, because there is a potential that male epithelial cells can, on senescence, appear as female epithelial cells. When you are dealing with cells, especially in an ejaculate, you're dealing with a dying population of cells. Depending on when the sample is taken, depending on how it was treated, you are going to see different morphologies, different metabolism, and different structures for the same cell.

Question: How many epithelial cells can one see in a normal ejaculate?

Chang: The number is not in the thousands, and certainly rarely in the hundreds in a normal population. But what I think you are most interested in is not the normal case, but the abnormal case because this is where the significance of the diagnosis or the analysis would be. It is in the abnormal case where you may have high numbers of epithelial cells in the male seminal plasma. We have seen patients with a 5 ml ejaculate that contained a half ml of epithelial cells. This is one extreme, but you can see that there are a large number of cells where normally you would see very few. In summary, in the normal case, one would see very few epithelial cells contributed by the male. In the abnormal case, one can see various degrees of epithelial cells arising from the male.

Divall: Could you state what those abnormalities are and discuss their frequency of occurence?

Chang: Again this is a clinical question. Dr. Sherins may be better able to answer it. Infection would be the most likely pathological situation. Often times, with epithelial cells, you see a high number of polymorphic nuclear leucocytes.

Question: In regards to differentiating female from the male epithelial cells, what's the capability for karyotyping them with regard to Barr bodies and Y chromosomes?

Chang: I can't say for sure. I have no experience in that matter. But that would be a nice approach. In fact, someone mentioned earlier this morning, I think it was Barry Brown, that one may want to determine the sex of a blood sample. And recently we have come across a person at Johns Hopkins University who has a label for the DNA of the Y chromosome; so, potentially from a blood sample he can determine, rather specifically, whether or not there is the presence of Y chromosome, which would definitely indicate the male.

Brown: I wanted to add a comment that would illustrate the identification of vaginal cells on the penis, thus indicating recent intercourse. A paper in the 1976 issue of the Journal of Forensic Sciences reported the identification of chromatin bodies in cells obtained from penile washings.

Chang: Again, I think the principle to stress is that you are dealing with heterogeneity, and there is a whole gradation of different kinds of cells. In dying, these cells express different rates of senescence, and so you see the whole gamat of changes, as well as morphologies. I think one must be able to anticipate these differences in order to consistently analyze seminal components.

Kearney: Would you say that the spermatozoa are fragile cells? What kind of conditions might lead to removal of the tail, or any alteration of the head that might cause abnormality?

Chang: I would have to answer that question with respect to spermatozoa of other animals. If you take the spermatozoa from a hamster, place them in a test tube, and shake the tube, you will decapitate a large portion of the cells. Now human cells are much more stable. It takes a lot of effort to break the heads or the tails if the cells are in semen. If the cells are out of semen, for example, if they have dried on a piece of clothing, then I would not be able to tell you anything about the stability of the cell. The nucleus of the mammalian sperm cell is one of the most stable structures on earth. This nucleus has a keratin-like structur with a lot of disulphide bonding, and it is rich in basi proteins which are not like proteins in the other cells c

the body. The characteristics of these proteins, in combination with disulphide bonds, make the sperm nucleus of most mammals, including humans, very stable. It would take chemical action, resulting in a reduction of these disulphide bonds, to break these nuclei apart. You can actually take a test tube full of human sperm and shake it very, very vigorously and not really see an increase in the amount of breakdown.

Kearney: It is common practice to use an extraction method for the removal of semen from cloth, using an aqueous solution of phosphate saline or distilled water. Would that process increase the fragile nature of a sperm cell, and are there solutions which would be more desirable to use in the extraction process?

Chang: That is a very interesting question, and I wish I had a specific answer for that. Not knowing how the spermatozoa would be affected by water, after having been dried on cloth, I would not be able to answer that question. In general, sperm cells can be dried, smeared on slides, and the structure will remain stable. Even without fixing, you can do a variety of enzymatic and chemical tests on it, and the sperm will still remain intact. Why you lose sperm when you try to extract them from cloth, I am not sure. They could be entrapped, or they could be bound through some chemical or ionic bonding. It is difficult to say.

Question: Can you expect a certain percentage of spermatozoa to be non-motile?

Chang: Yes, and that is part of the heterogeneity. Non-motile doesn't mean nonviable. Sperm can be totally viable, but not moving. Again if I can refer to Dr. Sherins studies on the "normal fertile population," a normal ejaculate is considered to have greater than 60 percent motile forms, which means that 40 percent are non-motile. In terms of numbers and proportions, even in the normal ejaculate, one would expect variations in morphology and motility as well.

Blake: During the course of this talk you emphasize the heterogeneity for morphology of spermatozoa. You don't mean to imply that there are problems with a technician seeing an abnormal spermatozoa and not identifying it as spermatozoa?

Chang: What I am saying is, that in training, the technician should be advised or educated as to the possibility of finding these abnormal sperm cells and identifying those cells as spermatozoa. Often when you see a sperm with a very abnormal morphology, it is easy to say this is not a sperm cell. If the person is prepared to identify these abnormal morphologies, then one can be more confident that the analysis will be correct.

Question: What is the normal pH range?

Chang: If you believe the literature, it is about 7.4 to 7.6 In practice, and we do thousands of semen analyses a year, it can range anywhere from 7 to 8.2. What is important is when the pH is measured. Because, if the semen sample is allowed to sit for a while, the pH goes up. If the pH of the semen sample is determined directly, then the pH reading would be more accurate. One thing I should mention is that semen, because of its very, very high concentration of protein (it can be as high as 60 to 80 milligrams per ml), is not a good sample for pH measurements. PH meters were not designed to be used in solutions containing high ionic strength or high amounts of protein. So pH is not a good determinant criterion for semen.

IDENTIFICATION AND PERSISTENCE OF SEMINAL CONSTITUENTS IN THE POSTCOITAL VAGINAL TRACT

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INTRODUCTION

The identification and characterization of semen in the vaginal tract of a rape victim constitutes the most important aspect of any scientific investigation of this crime. A great deal of time and effort has been devoted to this subject and it is most pertinent, therefore, to remind ourselves of what we are trying to achieve. In my view, we are trying to answer three questions. These are:

1. Has sexual intercourse taken place?
2. When did it occur?
3. With whom did it occur?

In this context the examination of vaginal contents is a matter of the detection, quantitation and characterization of semen. The procedures used at the Metropolitan Police Forensic Science Laboratory (MPFSL) for this purpose will be described and discussed.

Taking Samples for Analysis

Samples of vaginal contents are taken during the victim's medical examination on plain sterile cotton swabs. No other form of swab is, in our opinion, suitable. Vaginal aspirates are rare. Three samples are normally taken. These are, in order, swabbings from the external, low and high vagina. Some recent work at our laboratory has shown that after 2 days there appears to be a greater chance of finding spermatozoa on cervical swabs than on vaginal swabs (Wilson 1982). It is hoped, therefore, that in cases where the assault is not reported for 2 days or longer, vaginal and cervical swabs will be taken.

After sampling, the swabs are sealed and stored frozen until they are examined at the laboratory. They are not allowed to air dry. This has the intended advantage of preventing contamination and preserving any seminal group factors.

The Preliminary Examination of Vaginal Swabs

The high vaginal swab would normally be examined first. If semen is found and satisfactory grouping results are obtained, then the low vaginal swab would not be examined. However, it is not infrequent, due to vaginal drainage, for there to be more semen on the low vaginal swab. In this instance we may obtain satisfactory grouping results from the low vaginal swab even though grouping of the high vaginal swab gave inconclusive results. The external vaginal swab would only be examined if neither of the internal swabs was found to be stained with semen.

The swab is removed from the freezer and the whole of the cotton/wool head is extracted in 1 ml distilled water. The cotton/wool is then removed and the extract is centrifuged and separated into sediment and supernatant. Microscope slides are prepared from the sediment in order to search for spermatozoa. The supernatant is used for acid phosphatase determination, grouping, etc. We favor total-swab extraction primarily because it renders a homogenous sample for grouping, etc., and also because all insoluble material (spermatozoa) is recovered at one time.

The Detection of Semen

In most people's view, the *prima facie* evidence that sexual intercourse has occurred will be derived from details of the victim's medical examination and the presence of semen on the vaginal swabs.

The sediment obtained from the total swab extract is resuspended in one or two drops of distilled water. Some of this is spotted onto a microscope slide and allowed to dry on a hot plate at approximately 40 ° C. The slide is then heat fixed by passing it quickly through a Bunsen flame and stained with haemotoxylin and eosin. The slide is then viewed at 400X magnifica-

the number of sperm according to the following notation:

+ + + + Many in every field
+ + + Many or some in most fields
+ + Some in some fields
+ Few in some fields
- No spermatozoa

Such estimates can be used to formulate an opinion as to when sexual intercourse occurred and to the likelihood of obtaining successful grouping results. Swabs are always searched for spermatozoa since it is not uncommon to find small numbers of them even though chemical tests for semen (acid phosphatase) are negative.

Chemical Tests for Semen

The identification of semen in the absence of spermatozoa is one of the perennial problems of forensic biology. Azoospermia (no spermatozoa in the semen) can arise for a variety of reasons. It may be permanent as the result of a vasectomy, or it may be temporary as can result from frequent sexual intercourse, alcoholism, etc. Whatever the reason, it has been estimated that spermatozoa will not be found in approximately 2 percent of swabs known (from chemical tests) to be stained with semen (Willott 1982).

In the absence of spermatozoa, several chemical tests are used to identify seminal fluid. The methods currently used at the MPFSL involve the examination of the swab extracts for acid phosphatase (AP), p30 and, occasionally, choline.

Seminal plasma contains very large quantities of acid phosphatase and for many years high levels of this enzyme on vaginal swabs have been considered strongly indicative of the presence of semen.

One method for its semiquantitative determination consists of placing one drop of the extract onto filter paper and spraying this with a buffered solution of sodium α-napthyl phosphate and Brentamine Fast Blue B. The substrate, sodium α-napthyl phosphate, is hydrolyzed to α-napthol which diazotizes with the Brentamine Fast Blue B to yield a purple azo-dye. The time it takes for the purple coloration to develop provides a guide to the amount of acid phosphatase present.

A major problem arises from the fact that vaginal fluid itself contains acid phosphatase, although in much lower levels than found in semen. For example, in one study, semen-free vaginal swabs displayed AP reaction times of 40 to 200 seconds; times under 65 seconds were relatively uncommon, the average being 90 to 100 seconds. A reaction time of less than 30 seconds was therefore considered a very good indication of the presence of semen (Davies and Wilson 1974). However, such tests only provide a crude estimate of the amount of acid phosphatase on a vaginal swab.

More accurate and more reliable methods for the quantitation of acid phosphatase have, therefore, been sought. For example, at our laboratory a quantitative enzymatic assay has been developed (Davies 1978; Allard and Davies 1979). This is based on the ability of acid phosphatase to hydrolyze p-nitrophenylphosphate. The product, p-nitrophenol, is yellow in alkaline solution and the absorbance at 400 nm is a measure of the level of acid phosphatase activity. A recent modification to this assay is to also measure the total protein concentration of the swab extract and to express the acid phosphatase levels in terms of units AP activity per mg total soluble protein.

Other laboratories have developed immunological assays (rocket immunoelectrophoresis, radioimmunoassay) for the quantitation of seminal acid phosphatase (SAP) (Baxter, 1973; Dorrill *et al.* 1982). Each may claim a particular advantage but, in my view, they have a common goal; that is, to measure the amount of acid phosphatase and to establish a level above which one can be reasonably sure of the presence of semen. Such a goal, of course, requires that we have sufficient data on the levels of acid phosphatase in pre- and postcoital vaginal fluids. This information is not only difficult to collect, but variations in the methods of sampling vaginal contents and in the techniques used to quantitate AP almost certainly dictate that each laboratory must collect and analyze such information for itself.

Some techniques have sought to exploit any differences that may exist between SAP and vaginal acid phosphatase (VAP). For example, an electrophoretic method which differentiates between SAP and VAP has been described (Wraxall and Adams 1974).

The Quantitation of Semen

Estimates of the amount of semen on vaginal swabs and a knowledge of the persistence of various seminal factors can be used to formulate an opinion as to when sexual intercourse occurred.

The amount of semen is estimated by noting the number of spermatozoa and determining the level of acid phosphatase. The procedures used at the MPFSL have been described previously.

The rate of loss of seminal constituents from the vagina is dependent on many factors. Using both donor and casework swabs, it has been found that there is a considerable range in the levels of acid phosphatase and number of spermatozoa at each time interval after

sexual intercourse (Davies and Wilson 1974; Davies 1978; Willott and Allard 1982) The ranges reflect, in part, the different levels of these constituents in the seminal ejaculate and the variation in vaginal drainage as a result of the donor's postcoital activity. The results show, however, that there is a reasonable chance of detecting spermatozoa up to 24 hours after intercourse, that they are likely to be found up to 48 hours after intercourse and that it is possible to detect them up to 6 days after intercourse. High levels of acid phosphatase can be detected up to 2 days after intercourse, but the amount has usually fallen below an acceptance baseline level by 12 hours after intercourse.

Although such variation exists, the data can be used as a guide to determine time after intercourse. For example, in one case it was suggested by defense counsel that the semen present on the victim's high vaginal swab was in fact her boyfriend's from when they had had sexual intercourse 3 days prior to the offense. The presence of large amounts of semen (+ + + sperm heads and an AP time of 10 seconds) was inconsistent with this suggestion.

The Characterization of Semen

Having detected and estimated the amount of semen on a vaginal swab, the next step is to characterize it by means of grouping. In this way we hope to include or exclude the possibility that the semen came from a particular person.

Our ability to individualize semen is very limited compared to the extent we can characterize a bloodstain. For example, we have the facilities to group a bloodstain in 15 different systems, but for semen the number is only 6—ABO, Le, Gm, GLO, Pep A and PGM. If this is translated into terms of frequency (for the British population) the least amount of information from a perfect bloodstain of unlimited size is approximately 1 in 800, whereas for semen it is 1 in 30.

The methods used for the detection of these genetic markers are shown in Table 1.

Table 1. METHODS USED FOR THE IDENTIFICATION OF GENETIC MARKERS IN SEMEN

System	*Method*	*Reference*
ABO	Absorption-inhibition and absorption-elution	Pereira and Martin (1976)
Le	Absorption-inhibition	Pereira and Martin (1977)
Gm	Absorption-inhibition	Khalap and Divall (1979)
PGM	Electrophoresis	Price *et al.* (1976)
	Isoelectric focusing	Sutton (1979)
		Divall and Ismail (1983)
GLO	Electrophoresis	Parkin (1977)
Pep A	Electrophoresis	Parkin (1981)

It should be noted that absorption-inhibition and absorption-elution are used in parallel for the detection of the ABH group substances. Furthermore, a series of dilutions is prepared from the sample and this is tested by both methods

Electrophoresis or isoelectric focusing (IEF) can be used for the analysis of PGM IEF has the advantages of greater discriminating power and that mixtures can be more easily recognized. However, the isozyme patterns are more prone to distortion since the high salt concentration distorts the pH gradient. This effect can be minimized by performing the IEF in ultra-thin gels (Divall and Ismail 1983) and by running several dilutions of the original swab extract.

Theoretically, the least amount of information to be obtained from a seminal stain is 1 in 30. It could be considerably more, but in practice these frequencies are rarely, if ever, attained. It is obvious that other factors come into play. So what are these problems and, in particular, what are the difficulties in grouping semen from the postcoital vagina?

We can begin to appreciate the difficulties when we consider what can happen to seminal constituents in the vagina. The effects generally fall into one or more of the following four categories: dilution, degradation, modification and mixing.

The Effect of Dilution

Unlike the direct formation of a blood or seminal stain on an item of clothing, the very act of depositing semen in the vaginal canal dilutes the seminal constituents. This effect is enhanced by continuous vaginal drainage. The rate of loss due to drainage is dependent on many factors—whether the victim lies down due to injury, runs around, bathes, douches the vagina, etc. Dilution will, of course, increase with elapsed time after intercourse and can be expected to affect individual seminal constituents to the same extent.

The effect of dilution is a simple one—the individual seminal group factors are soon reduced to a level beyond the detection limit of the grouping tests. It might be felt, therefore, that our efforts should be directed at increasing the sensitivity of the grouping tests.

One of the approaches we have tried is to concentrate the swab extract by freeze-drying or ultrafiltration. This has met with some success. For example, in comparative trials, freeze-drying the swab extract has produced a considerable enhancement of the seminal PGM isozyme bands after starch gel electrophoresis. This effect is illustrated in Figure 1. Sample 6 was a portion of a vaginal swab inserted directly into the gel, sample 7 was the swab extract and sample 8 was the

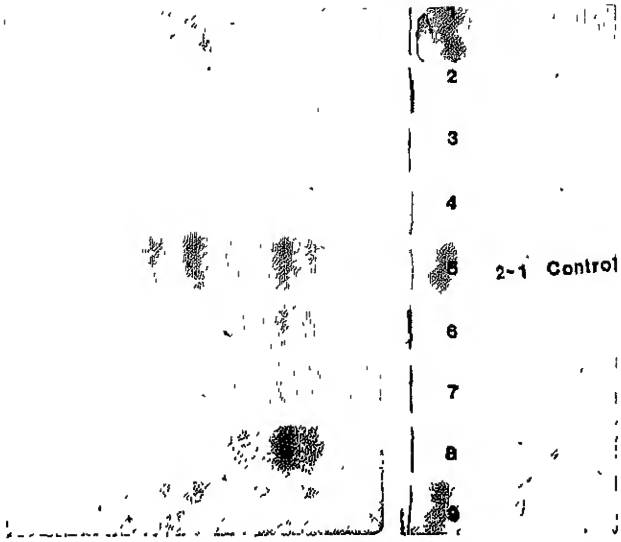

Figure 1. Starch gel electrophoresis of PGM illustrating the effect of concentrating a vaginal swab extract. Sample 6 was a portion of a vaginal swab, sample 7 was the swab extract and sample 8 was the freeze-dried extract reconstituted in a small volume of distilled water. The donor was PGM-1 and her sexual partner, PGM 2-1.

reconstituted freeze-dried extract. The donor was PGM 1 and her sexual partner, PGM 2-1.

Such steps, however, are not always advantageous. Extract concentration increases the ability to detect vaginal group factors, but it can produce considerable distortion of the isozyme patterns after electrophoresis and IEF due to a high salt concentration. Finally, the small amounts of ABH group substances which can be detected in the body fluids of nonsecretors, especially by sensitive methods such as absorption-elution, can be concentrated to the extent that they are readily detected by both absorption-inhibition and absorption-elution. The levels of ABH group substances as assessed by these methods therefore become meaningless with regard to ascertaining secretor status.

The Effects of Degradation

Degradation may be defined as the active breakdown and loss of seminal group factors in the postcoital vagina. The pathways and mechanisms remain very unclear. We know that semen itself undergoes complex changes after ejaculation, associated in part with a coagulation-reliquifaction process. Such changes generate powerful proteolytic activity which could contribute to the loss of seminal group activity in the vagina.

A further and perhaps more important consideration is the effect of vaginal secretions on semen. It is becoming very clear that the biochemical properties of the postcoital vagina are different from the precoital vagina. It has been postulated, for example, that the rapid loss of seminal glyoxalase (GLO) results from the action of postcoital vaginal proteolytic activity (Parkin 1977). Degradation is an active process and each seminal constituent can be expected to be lost at a characteristic rate.

The effects of dilution and degradation are the major limiting factors to the successful detection of any seminal constituent in the postcoital vaginal tract. From donor swabs it has been estimated that seminal ABH antigens should be detected up to 24 hours after intercourse (Davies and Wilson 1974), seminal PGM up to 6 hours after intercourse (Willott 1982), and seminal GLO up to 2 hours after intercourse (Parkin 1977). There is, however, considerable variation between individual donors and this must be considered when evaluating grouping results.

The amount of semen on a vaginal swab, as assessed by the number of spermatozoa and AP level, is often used as a guide to the likelihood of obtaining successful grouping results and, thus, to deciding whether or not grouping tests should be attempted. There is, however, considerable variation as illustrated by the following somewhat exceptional example. A girl (group O secretor) was raped and her vaginal swabs, taken 8 hours after the offense, were examined for semen and grouped in the ABO system. The results are shown in Tables 2 and 3. It can be seen that there was very little semen on the swabs, but both of them gave good A secretor reactions. A possible explanation for these results is that the assailant did not ejaculate, but sufficient quantities of pre-ejaculate secretions, devoid of or low in sperm and acid phosphatase, were deposited in the victim's vagina during intercourse.

The Effects of Modification

Modification may be defined as an induced change in a seminal group factor such that rather than its mere disappearance, it manifests itself as an altered but detectable moiety in our grouping tests. Such changes are more problematic than degradation because the altered

Table 2. ANALYSIS OF VAGINAL SWABS FOR PRESENCE OF SEMEN. VICTIM: O SECRETOR

	Low Vaginal Swab	*High Vaginal Swab*
Spermatozoa	1 head	None
AP time	+ at 20 seconds	- at 30 seconds
	strong at 2 minutes	medium + at 2 minutes
Grouping	A secretor	A secretor

Table 3. ABO GROUPING REACTIONS OBTAINED FROM VICTIM'S LOW AND HIGH VAGINAL SWABS. THE VICTIM WAS GROUP 0 SECRETOR

	Inhibition			Elution		
	A	*B*	*H*	*A*	*B*	*H*
LOW VAGINAL SWAB						
N	–	4	4	3 +	–	4
5	–	4	4	3 +	–	3 +
10	–	4	4	3 +	–	2 +
20	–	4	4	3 +	–	+
40				3	–	–
HIGH VAGINAL SWAB						
N	–	4	4	4	–	–
5	–	4	4	4	–	–
10	–	4	4	4	–	–
20	–	4	4	4	–	–
40				4	–	–

form may appear to represent a different phenotype. Such changes are confined, as far as we know, to the glyoxalase system. Vaginal swabs taken within 2 hours of intercourse show the isozyme pattern expected from the phenotype of the seminal glyoxalase. However, some swabs taken between 2 and 7 hours after intercourse show a GLO pattern which cannot be recognized as the phenotype of the semen or the phenotype of the female donor. It has been suggested that these patterns are produced, in part, by the action of vaginal proteolytic enzymes on seminal glyoxalase (Parkin 1977). This gives rise to GLO isozymes with an altered electrophoretic mobility.

The Effect of Mixtures

The interpretation of grouping reactions obtained from a mixture of two or more body fluids is a difficult subject.

Vaginal secretions and, more relevantly, postcoital vaginal secretions are by no means lacking in their own content of group factors. Most workers recognize that high levels of ABH group substances can be found in the vaginal secretions of secretors throughout the menstrual cycle (Martin 1981). The presence of vaginal ABH substances on postcoital vaginal swabs can and must, therefore, always be assumed. The GLO, PGM, and Gm factors occur in low concentrations or are undetectable in precoital vaginal secretions but are found in significantly elevated amounts postcoitus.

Five out of six genetic markers found in semen (ABH, Le, Gm, PGM and GLO) are known to be detectable in postcoital vaginal secretions. These will reflect the blood group of the female concerned and all grouping reactions obtained from vaginal swabs must be interpreted in the light of a contribution made by any semen and a contribution by the vaginal secretions. With this in mind, it is possible to recognize three types of combinations.

First, there is the situation where the groups of semen and vaginal secretions are the same. Here, any grouping reactions could be attributed either to the semen or the vaginal secretions. Approximately 29 percent (ABO) and 48 percent (PGM) of victims will be raped by a man of the same group (Davies 1982).

The second effect is masking, where the group of the victim masks or hides that of the assailant's semen. This effect will be observed in 33 percent (ABO) and 23 percent (PGM) of cases. Masking, however, is dependent on the relative proportions of semen to vaginal secretions and is not always complete. A typical example is shown in Table 4. The victim was a group A secretor and her vaginal secretions could be expected to mask group 0 secretor semen. However, the inhibition and elution with anti-H is in far excess of that observed with anti-A and it is possible to conclude the presence of group 0 secretor semen in this instance.

Table 4. ABO GROUPING REACTIONS OBTAINED FROM THE VICTIM'S HIGH VAGINAL SWAB. VICTIM WAS GROUP A SECRETOR

	Inhibition			Elution		
	A	*B*	*H*	*A*	*B*	*H*
N	2 +	4	–	2 +	–	4
5	3	4	–	3 +	–	4
10	4	4	–	3	–	4
20	4	4	1	1	–	3 +
40				–	–	3

A similar situation can arise in the PGM system if careful attention is paid to the relative intensities of the component isozyme bands. This effect is illustrated in Figure 2. Sample 6 is a donor's semen-positive high vaginal swab. The swab gave all four PGM_1 isozyme bands, but it can be seen that the a and c bands were much stronger than the b and d. This pattern of intensities is not seen with a normal PGM 2–1 and in this instance it is possible to conclude the presence of PGM 1 semen. The donor was PGM 2–1 and her sexual partner, PGM 1.

Analysis of PGM by isoelectric focusing can also give clear indications of a mixture. For example, a vaginal swab from a PGM (1+1–) victim was positive for semen and gave a PGM (2–,1+,1–) result. Only

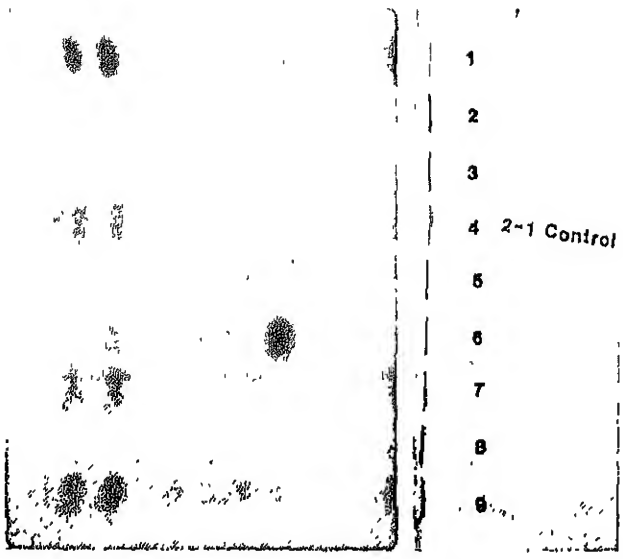

Figure 2. Starch gel electrophoresis of PGM. Sample 6 was a semen-positive high vaginal swab. The donor was PGM-2 and her sexual partner, PGM 1.

the 2 – isozyme could not have originated from the victim. The semen could, therefore, have originated from a man who was PGM (2 –), (2 – 1 +) or (2 – 1 –).

The third and final effect is when the combination of groups is such that it is possible to group the semen.

Finally, it must be noted that when grouping a mixture of vaginal material and semen, it is possible to obtain the ABO group of one component and the PGM group of the other (Davies 1982). For example, the victim is group A, PGM 1; the assailant is group B, PGM 2; and the victim's vaginal swab gives reactions for group B, PGM 1.

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DISCUSSION

Question: Do you concentrate the extract from your vaginal swabs for electrophoretic analysis?

Divall: The answer is simply this. We use the extract. I am well aware that sometimes it is diluted to such an extent that we fail to pick up the enzyme activity. We are well aware of that, and that is why we

have looked into this possibility of concentrating by freeze-drying or ultrafiltration. But, of course, this is why I must emphasize that this is only a suggested way of approaching it. We don't necessarily have to stick to this. What some people might choose to do is to make a total extract of the high vaginal swab; they will find plenty of spermatozoa there. They will then take a low vaginal swab; of course, they have to take a little bit off to look for the spermatozoa. They would then perhaps use the neat swab by cutting a little piece out to use as an electrophoretic insert. But the overdilution or loss of the enzymes, by the approach that I described, is a problem, and we are trying to do something about it.

Question: Have you looked at esterase D in seminal fluid?

Divall: Yes, we have made a preliminary investigation of esterase D in seminal fluid. I have to add here that we are now using isoelectric focusing of esterase D, in which we detect 6 phenotypes instead of the normal 3 detected by starch gel electrophoresis. We have used this particular assay, obviously for blood mainly, but another thing we have done is to look at vaginal swabs and some semen samples. On vaginal, semen-free swabs we failed to detect any esterase D activity. On those that have been stained with semen, and those seminal samples that we have run, and I must point out that the number is very small, we've detected some esterase activity. Of course, the patterns are distorted, and we have yet to sort out whether it is esterase D activity or whether it represents the phenotype of the girl in question.

Lincoln: In the case where you described the victim as being blood group A, PGM 2 and the suspect as being PGM 1, you said you were not finding the A activity of the victim, and that the reaction was confined to the PGM 2 of the victim. It seems to me, that another explanation might be that the PGM 2 could be from the semen of some other person.

Divall: I think we are always placed in this position when trying to get useful information from casework. We don't ever really know; we might have the wrong guy. All I can say is that our approach under these circumstances, when we choose these types of examples, is that the total collection of evidence is so overwhelming that we say we think this is the guy. The police then uses this information to prosecute and to send the person to jail. And, of course, also we have observed these phenomena with donor swabs.

Question: Are we referring to esterase 5?

Divall: Yes we are.

Question: When you were talking about what you should see, such as spermatozoa or acid phosphatase, were you saying that if you don't see it, you can make a statement that intercourse did not occur?

Divall: Most certainly not! That is not the implication that we make, and it is never really taken like that by the courts in the United Kingdom. The results of our tests are simply negative. That does not imply anything other than that we have failed to find the material.

Question: You indicated that the PGM activity lasts 6 hours postcoitus, and glyoxalase lasts 2 hours; have we any information on peptidase A?

Divall: The results we have indicated that peptidase A survives for about 2 hours. However, that is based upon the assumption that we are failing to detect peptidase A in the postcoital vagina. When we take vaginal swabs from the donor in half an hour, 1 hour, and 1½ hours, we pick it up. But of course in the UK, everyone is type 1, so one perhaps could suggest that it is possible that peptidase A is wiped out in a quarter of an hour, and what you are picking up is from the vagina. Perhaps someone can answer whether or not peptidase A occurs in postcoital vaginal secretion.

Blake: We have found peptidase A on precoital swabs.

THE ACID PHOSPHATASE TEST

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INTRODUCTION

The detection of semen is of concern in two principal contexts: (a) in the analysis of swabs or washings collected from the vagina or other orifices of sexual assault victims; and (b) in the analysis of suspect stains. In most cases, the presence of semen can be established by the detection of sperm. However, sperm searches are impractical or inadequate in a number of situations, such as in surveying clothing or bedding for semen stains. Moreover, reliance on sperm detection for the identification of semen is misplaced in cases involving males who have few or no sperm in their semen due to genital dysfunction or vasectomy. Finally, there are cases in which the presence of semen is strongly suspected, yet the sperm search is inconclusive. These situations illustrate the need for alternative methods for the detection of semen.

The most venerable of the alternative tests for semen is the acid phosphatase test. The presence of very high levels of acid phosphatase activity in semen was first reported in 1935 by Kutscher and Wohlbergs; this activity was found to originate in the prostate gland which itself contains extraordinarily high levels of acid phosphatase activity. Medico-legal utilization of this acid phosphatase activity for the detection of semen was established shortly thereafter (Lundquist 1945; Rasmussen 1945; Hansen 1946; Riisfeld 1946). Since then, substantial literature has accumulated on the forensic application of the "acid phosphatase test"; this literature has been reviewed by Kind (1964) and by Sensabaugh (1982). Prostatic acid phosphatase is also of considerable clinical interest; serum levels of acid phosphatase are often elevated in patients with prostatic cancer and other diseases. The extensive literature on clinical applications has been reviewed by Bodansky (1972), Yam (1974), and at a recent symposium of the New York Academy of Sciences (Shaw *et al.* 1982a). Much of the clinical literature is relevant to forensic concerns, notably with regard to the tissue specificity of acid phosphatase and to assay standardization.

The objective of this review is to outline what we know, what we think we know (but aren't really sure about), and what we don't know about the acid phosphatase test. The review is divided into three main parts. The first part provides a sketch of the basic biology and biochemistry of the acid phosphatase activity found in semen and surveys methods for its detection. The second part addresses the question of specificity. The third part is concerned with the factors that determine acid phosphatase levels in evidence materials. It is hoped that this approach, defining what we don't know as well as what we do know, will stimulate further thinking and research.

BASIC BIOLOGY, BIOCHEMISTRY, AND METHODS OF DETECTION

Tissue Origin

As previously noted, the acid phosphatase in human semen originates from the prostate gland. There is no evidence that the testes or seminal vesicles contribute any acid phosphatase activity to semen. The seminal enzyme is thus often referred to as "prostatic" acid phosphatase.

The enzyme is synthesized in the prostate in secretory epithelial cells and is secreted from these cells into the prostatic gland ducts. Histochemical analysis of prostatic tissue shows acid phosphatase activity localized both in secretory granules contained in the secretory cell cytoplasm and in the lumina of the gland ducts. The prostatic gland ducts empty into the urethra (Aumuller 1979).

The synthesis and secretion of high levels of acid phosphatase by the prostate is under androgen control. The enzyme first appears at significant levels in prostatic secretions at puberty, remains at high levels from ages about 15 to 40, and declines gradually thereafter (Kirk *et al.* 1952; Mann and Lutwak-Mann 1981). The mechanism of androgen regulation is not known but probably follows the general pattern of steroid hormone-regulated protein synthesis (Williams 1981).

In view of recent reports of a "urethral ejaculate" in females containing "prostatic" acid phosphatase activity (Addiego *et al.* 1981), it is of note that no histochemically detectable acid phosphatase is present in the female periurethral ducts, the embryological analog of the male prostate (Nadji *et al.* 1980).

Biochemical Properties

The acid phosphatase found in prostatic tissue and in semen is technically classified along with other acid phosphatases as orthophosphoric monoester phosphohydrolase (acid optimum) E.C. 3.1.3.2.

The enzyme has been purified both from prostatic tissue and from seminal plasma. The biochemical and enzymatic properties of the purified enzymes will be only summarized here; for more detail, reference should be made to appropriate review articles and the references cited therein (Hollander 1971; Yam 1974; Harris and Hopkinson 1976; Sensabaugh 1982; Van Etten 1982; Lam *et al.* 1982).

The acid phosphatase found in semen is a glycoprotein of 100,000 to 120,000 dalton molecular weight. The protein is a dimer consisting of two subunits of approximately 50,000 to 55,000 dalton molecular weight. It is presumed the two subunits are identical. The variation in the molecular weight estimates may be due to inherent ambiguities associated with molecular weight determination of glycoproteins. In addition, there may be true variation in the enzyme molecular weight due to differences in the amount of carbohydrate bound. Indeed, a recent report suggests that the semen enzyme may exist in two molecular weight forms (100,000 daltons and 120,000 daltons) differing in carbohydrate content (12 and 20 percent, respectively) (Liu *et al.* 1983). These forms may be the same as those described earlier by others (Mattila 1969; Ostrowski *et al.* 1970).

The bound carbohydrate is high in mannose which suggests that the protein contains one or more N-asparagine-linked oligosaccharide units (Kornfeld and Kornfeld 1980). It is not known whether the protein also contains O-serine-linked polysaccharide.

The bound carbohydrate also includes 5 to 10 residues of sialic acid per enzyme molecule. This bound sialic acid contributes substantially to the charge heterogeneity of the enzyme. Characterization of the enzyme by isoelectric focusing shows as many as 20 isozymes with isoelectric points in the range 4.5 to 5.5 (Ostrowski *et al.* 1970; Sutton and Whitehead 1975; Toates 1979); this isoelectric heterogeneity can also be demonstrated by electrophoresis under certain conditions (Figure 1) (Smith and Whitby 1968). Removal of sialic acid residues by treatment with neuraminidase reduces this heterogeneity; the "ladder" pattern of bands collapses to one band or a few bands with higher isoelectric points. Sialic acid removal appears to have no significant effect on enzyme stability or enzyme activity (Dziembor *et al.* 1970).

Despite the various kinds of molecular heterogeneity noted above, all the acid phosphatase isozymes in

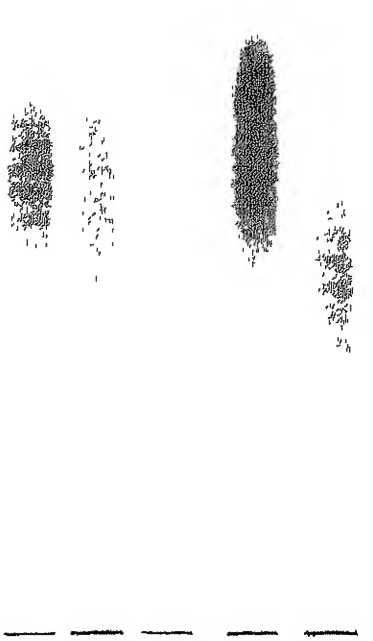

Figure 1 Electrophoretic heterogeneity of the acid phosphatase in semen. Shown are the isozyme pattern of 5 semen samples (diluted 1:100) following starch gel electrophoresis in a pH 5.9 Tris-citrate buffer system (Sensabaugh 1975). Isoelectric focusing gives similar multiple-band patterns.

semen appear to be immunologically identical. Removal of sialic acid residues does not alter this identity (Inman 1978). These observations provide evidence that the oligosaccharide structures bound to the enzyme are not involved in the immunological reactivity of the enzyme. If this is so, then the immunological identity of the seminal acid phosphatase isozymes is good evidence that these isozymes have a common genetic origin.

The enzymatic properties of the prostatic acid phosphatase have been well studied. The optimal pH for enzyme activity is about pH 5.0, although there is some variation for different substrates. The enzyme has a rather broad substrate specificity; most natural and synthetic phosphate monoesters will serve as substrates. A characteristic catalytic feature of the enzyme is its inhibition by L(+) tartrate; tartrate also inhibits lysosomal acid phosphatases but does not inhibit the low molecular weight acid phosphatase from red cells nor any of the alkaline phosphatases.

Despite all that is known about the acid phosphatase from the prostate, we would greatly benefit from additional knowledge. In particular, more detailed knowledge of the structure of the enzyme and its attached oligosaccharide units would give us better insight into the determinants of enzyme stability, the origin of the

enzyme's heterogeneity and, ultimately, the basis of genetic specificity

Methods of Detection

1. *Enzyme activity.* Acid phosphatase is most easily detected by its enzyme activity. A number of substrates have been employed in clinical assays for prostatic acid phosphatase, including phenyl phosphate, β glycerol phosphate, p-nitrophenol phosphate, α naphthyl phosphate, and thymolphthalein phosphate (Bowers 1982). None of these, however, is completely specific for the prostate enzyme. All are also substrates for lysosomal acid phosphatases found in most tissues. In addition, p-nitrophenol phosphate and phenyl phosphate are also good substrates for the low molecular weight acid phosphatase found in red cells and other tissues. For the most part, whatever specificity has been claimed for these particular substrates has a quantitative basis; that is, assay conditions have been selected so that clinically insignificant levels of acid phosphatase activity fall in the background activity range. There has been a recent report that acid phosphatase purified from the prostate reacts with choline o-phosphate whereas a purified lysosomal acid phosphatase from liver does not (Saini and Van Etten 1978 a, b); this substrate has not as yet received clinical evaluation.

The inhibition of prostatic acid phosphatase by L-tartrate was accepted for many years to be a specific character of the prostatic enzyme. Several commercial clinical assays for "prostatic acid phosphatase" assess the target enzyme as the difference between total acid phosphatase activity and acid phosphatase activity in the presence of tartrate. However, it is well established that other tissue acid phosphatases are also tartrate inhibitable (Abul-Fadl and King 1949; Kilsheimer and Axelrod 1958; Yam 1974). Thus, tartrate inhibition is a specifying trait only relative to nontartrate-inhibited acid phosphatases such as the low molecular weight red cell enzyme. It is to be noted that because the red cell and prostatic enzymes do differ in this regard, the two activities can be distinguished in semen-blood mixtures.

In the forensic realm, both qualitative and quantitative tests for acid phosphatase have been employed. Qualitative spot tests for acid phosphatase have been well reviewed by Kind (1964). They generally employ α naphthyl phosphate as the substrate; the α naphthol released by the enzyme reaction is detected by its reaction with a diazo dye which produces an intensely colored and often insoluble product. Several diazo compounds have been used for the post-enzyme-coupling reaction; they obviously contribute nothing to the inherent specificity of the enzyme reaction and there is little to differentiate among them provided they give a good strong color reaction. Interestingly, several feminine hygiene products appear to contain phenols or naphthols which react with the diazo coupling agents (Brown and Brown 1974). The qualitative spot tests have been used to semiquantitate acid phosphatase activity by measuring the time of reaction required to produce a specified color intensity (Davies and Wilson 1974); there is, however, little to recommend this kind of semiquantitative assay given the precision and convenience of standard quantitative assays.

Forensic application of quantitative assays has been mainly in the area of postcoital vaginal swab analysis. Thymolphthalein phosphate and p-nitrophenyl phosphate appear to be the favored substrates.

2. *Immunological detection.* Several research groups have offered evidence over the past two decades that antibodies against prostatic acid phosphatase were immunologically unreactive with acid phosphatases in other tissues (Shulman *et al.* 1964; Ablin *et al.* 1970; Vihko *et al.* 1978b; Chu *et al.* 1978). These findings prompted research to develop specific and sensitive immunoassays for the prostatic enzyme. Leaving the specificity issue aside for later discussion, three immunoassays will be described here.

Prostatic acid phosphatase remains active when bound by an antibody (Shulman *et al.* 1964). This provides the basis for both qualitative and quantitative tests in which an enzyme captured by an antibody is detected by its activity; the specificity of the immunological reaction is coupled to the sensitivity of the enzyme assay. At the simplest level, this feature can be exploited to detect immune precipitates following Ouchterlony double diffusion or counterimmunoelectrophoresis (Baxter 1973). A more sophisticated application employs the enzyme reaction to quantitate acid phosphates captured by an immobilized antibody (New England Immunology Associates 1982).

The second immunoassay of note is rocket immunoelectrophoresis (Baxter 1973). Prostatic acid phosphatase is a good candidate for this kind of assay since it has a low isoelectric point and hence good anodal mobility at the pH of the electrophoresis. Rocket electrophoresis is essentially a quantitative assay; the height of the "rocket" is correlated to the amount of immunoreactive protein.

The third, and most sensitive of the immunoassays, is radioimmunoassay (RIA) (Foti *et al.* 1977; Shaw *et al.* 1981). RIA methods for acid phosphatase have gained clinical acceptance and RIA kits are commercially available. Forensic application has been limited heretofore to laboratories with radioisotope facilities. However, as enzyme-linked immune adsorbant assay

(ELISA) methods of comparable sensitivity become available, this immunoassay will become accessible to any laboratory. RIA and ELISA systems are particularly suited for monoclonal antibodies and whatever advantages might be provided by anti-acid phosphatase monoclonals will surely exploit these assay systems.

3. *Electrophoretic detection.* Tissue phosphatases were among the first group of enzymes to be characterized electrophoretically. These early studies indicated considerable variability in banding patterns from tissue to tissue and species to species (Lundin and Allison 1966). Forensic applications have focused on differentiation of the acid phosphatases from semen, vaginal fluid and nonhuman sources. These acid phosphatases have been found to vary in electrophoretic mobility and in isoelectric focusing patterns, the complexity of the acid phosphatase isozyme patterns depending on the method used (Anzai 1964; Walther 1971; Adams and Wraxall 1974; Sutton and Whitehead 1975; Blake 1976; Stolorow *et al.* 1976; Inman 1978; Toates 1979). However, these methods do not provide the hoped for unequivocal qualitative differentiation of acid phosphatases from different sources. The reasons for this will be outlined in the following section.

THE QUESTION OF SPECIFICITY

Acid phosphatase activity is found in animals, plants and microbes (Hollander 1971). Given this ubiquity, it is pertinent to ask how well seminal acid phosphatase activity can be distinguished from acid phosphatase activity originating from other sources. The answer to this question depends on the answer to a more fundamental question: the biochemical and genetic relationship of the acid phosphatase enzymes of concern. This section addresses the specificity issue in terms of two practical questions: (a) the differentiation of seminal and nonhuman acid phosphatase activities, and (b) the relationship of the acid phosphatase in semen to the acid phosphatases in vaginal fluids and other human tissues.

Differentiation of Seminal and Nonhuman Acid Phosphatases

The high acid phosphatase activity in semen distinguishes this source from almost all nonhuman sources. Kind (1964) noted that several plants including cauliflower, malerblume and gorse give strong acid phosphatase reactions; quantitative data were not given so direct comparison with semen levels cannot be made.

Qualitative differentiation of seminal acid phosphatase from nonhuman acid phosphatases can be made by tartrate inhibition tests, by electrophoresis, or by immunological tests. Many plant and microbial acid phosphatases are not inhibited by L + tartrate and hence, are readily distinguished from the tartrate-inhibited seminal enzyme (Kilsheimer and Axelrod 1958). Tartrate inhibition, however, is a characteristic property of the lysosomal acid phosphatases found in animal tissues; the differentiation value of tartrate inhibition is thus limited. Electrophoresis can differentiate many nonhuman acid phosphatases from the seminal enzyme (Lundin and Allison 1966; Walther 1971; Adams and Wraxall 1974). The limitation of electrophoretic differentiation is that a nonhuman acid phosphatase might by chance have the same electrophoretic mobility as the seminal enzyme. Immunological testing provides the most fundamental approach to species differentiation; given what is known of species variation in protein structure, nonprimate acid phosphatases would not be expected to react with antibody to the human seminal enzyme (Wilson *et al.* 1977). Both anti-human semen and anti-prostatic acid phosphatase antisera are commercially available.

The Relationship of the Acid Phosphatase in Semen and the Acid Phosphatase in Vaginal Fluids

The acid phosphatase in vaginal fluids has not been characterized to the extent of the seminal enzyme. Nevertheless, it is clear that the two have very similar properties. The vaginal enzyme has a molecular weight of about 100,000 daltons, the same as the seminal enzyme (Blake 1976). Both are tartrate inhibitable (Willott 1972). As shown in Table 1, the two have essentially identical specificity profiles with respect to the commonly used substrates. Immunological testing indicates that the vaginal enzyme reacts with anti-prostatic acid phosphatase and by Ouchterlony analysis the two are indistinguishable (Baxter 1973; Inman 1978). This latter finding indicates that the protein structures of the two enzymes are very similar.

Table 1. SUBSTRATE SPECIFICITIES OF ACID PHOSPHATASES FROM SEMEN AND VAGINAL FLUIDS

Substrate	*ACP Source*	
	Semen (5)	*Vaginal Fluids (5)*
p Nitrophenyl Phosphate (2mM)	1.0	1.0
Phenyl Phosphate (2mM)	0.129	0.133
Methylumbelliferone Phosphate (2mM)	1.007	1.164
Thymolphthalein Phosphate (5mM)	0.856	0.869
alpha Naphthyl Phosphate (2mM)	0.473	0.569

All assays run in 0.1 M citrate pH 5.5 Activity levels are reported relative to the p-nitrophenyl phosphate activity.

The only difference that has been found between the two enzymes is their electrophoretic behavior. Differences in electrophoretic mobility show up in several electrophoretic systems and by isoelectric focusing (Anzai 1964; Adams and Wraxall 1974; Sutton and Whitehead 1975; Blake 1976; Stolorow *et al.* 1976; Inman 1978; Toates 1979). Figure 2 illustrates a prototypic separation of the seminal and vaginal isozymes by acrylamide gel electrophoresis at pH 8.8; the activity bands are well separated with the semen band exhibiting the greater negative charge. Although it does not show up in this electrophoretic system, each of the activity bands is in fact a compressed cluster of bands.

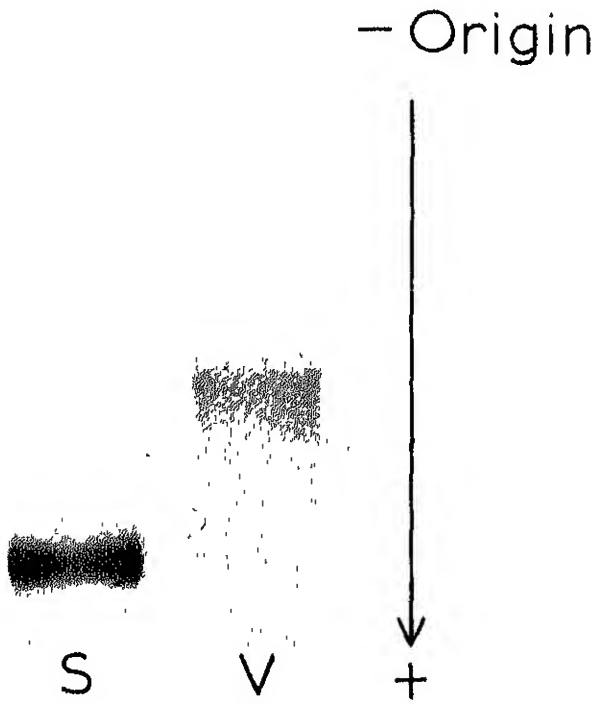

Figure 2. Electrophoretic differentiation of acid phosphatases from semen (S) and vaginal fluids (V). Electrophoresis was done on an acrylamide gel in a Tris-glycine pH 8.8 buffer system. (Reprinted from Sensabaugh 1982).

out by isoelectric focusing and by electrophoresis in other systems (note Figure 1); in these systems, the seminal and vaginal band patterns overlap in part and are not as well resolved.

The electrophoretic differentiation of the vaginal and seminal enzymes, however, is not as straightforward as Figure 2 might suggest. Some semen-free vaginal fluid samples show multibanded isozyme patterns that overlap the semen band (Figure 3, left). More significantly, casework samples sometimes exhibit idiosyncratic isozyme patterns containing multiple bands or bands migrating in between the prototype vaginal and seminal bands (Figure 3, right). The practical consequence of these idiosyncracies is that seminal and/or vaginal origin cannot be reliably inferred unless the observed patterns are prototypic.

The occurrence of these variant patterns suggests that the biochemical differences between the two enzymes are secondary rather than primary. Differences in carbohydrate content could easily account for the variable electrophoretic mobilities. Indeed, desialidation of seminal acid phosphatase changes its mobility to the vaginal band position. These observations point out that the relationship of the seminal and vaginal acid phosphatases is fundamentally a genetic question. If the two enzymes are products of different genetic loci, then differences between them would be intrinsic and stable. On the other hand, if the two are products of the same locus and differ only as the result of a secondary modification, then reliable differentiation depends on the stability of the modification.

The Relationship of the Acid Phosphatase in Semen to Other Human Acid Phosphatases

Biochemical characterization of the acid phosphatases in human tissues indicates the existence of two major enzyme classes: a high molecular weight class and a low molecular weight class. The distinguishing properties of these two classes are shown in Table 2;

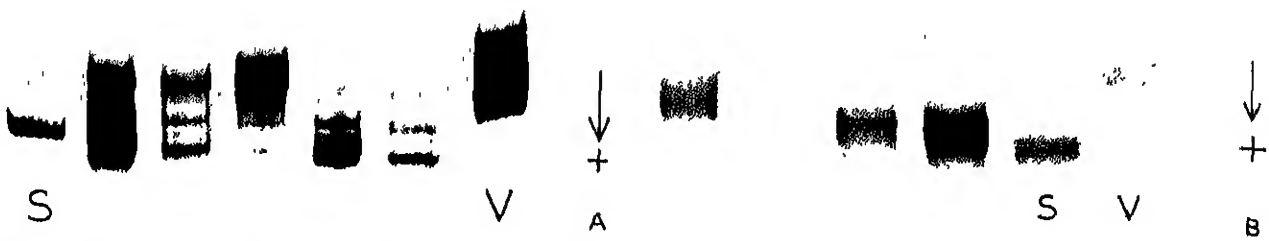

Figure 3. Anomalous acid phosphatase isozyme patterns from vaginal fluids and case samples. Vaginal fluid standards (V) and semen standards (S) are indicated; electrophoresis was as in Figure 2. (Left) Vaginal fluid samples from a female abstaining from coitus; samples were collected on different days of her cycle. (Right) Vaginal swab samples from rape victims; note the bands intermediate between the semen and vaginal band positions (Irman 1979). (Reprinted from Sensabaugh 1982)

Table 2. ENZYMATIC AND MOLECULAR PROPERTIES OF THE TWO MAJOR CLASSES OF TISSUE ACID PHOSPHATASES, PROSTATIC ACID PHOSPHATASE AND VAGINAL FLUID ACID PHOSPHATASE

	Tissue acid phosphatase class[a]			
	High MW	*Low MW*	*Prostate, semen*[b]	*Vaginal fluids*[c]
Subcellular localization	lysosomes, perhaps microsomes	cytoplasm	secretory vesicules in prostate	
Molecular properties				
Molecular weight	90,000–125,000 d	15,000 d.	90,000–110,000 d.	ca. 100,000 d
Subunit structure	dimer	monomer	dimer	N.D
Bound carbohydrate	yes	no	yes	yes
Substrates				
p-nitrophenyl phosphate	+	+	+	+
α-naphthyl phosphate	+	–	+	+
thymolphthalein phosphate	+	–	+	+
β-glycerol phosphate	+	–	+	+
Inhibitors				
formaldehyde	–	+	–	–
L(+) tartrate	+	–	+	+
Genetic Locus	ACP_2, ACP_3	ACP_1		

[a]Abul-Fadl and King 1949; Kilsheimer and Axelrod 1958, Neil and Horner 1964; Nelson 1966, Di Pietro and Fengerle 1967, Brightwell and Tappel 1968; Ide and Fishman 1969; Li *et al.* 1970, De Duve 1969; Swallow and Harris 1972; Sensabaugh 1975; Rehkop and Van Etten 1975, De Araujo *et al* 1976, Saini and Van Etten 1978a, 1978b

[b]Abul-Fadl and King 1949; Smith and Whitby 1968; Derechin *et al* 1971; Roy *et al.* 1971, Bodansky 1972; Lam 1973; Luchter-Wasyl and Ostrowski 1974; Yam 1974; Ostrowski *et al.* 1976, Vihko *et al.* 1978, Vihko 1978

[c]Willott 1972; Blake and Sensabaugh, unpublished

the two classes clearly differ in their enzymatic and molecular properties. They also have different subcellular localizations; the high molecular weight enzymes are found in the lysosomal organelles within cells and the low molecular weight enzymes are found in the cell cytoplasm. As indicated in the table, the acid phosphatases in semen and in vaginal fluids possess the general suite of catalytic and molecular properties that define the high molecular weight class and clearly belong to this class.

The high molecular weight acid phosphatases exist in multiple electrophoretic forms in most tissues and there is good genetic evidence implicating the existence of at least two distinct structural gene loci. The genetic picture has been defined in terms of the isozyme nomenclature of Beckman and Beckman (1967). In their electrophoretic system, the high molecular weight acid phosphatases migrate anodally and separate into four major bands of activity, designated A, B, C and D, from anode to cathode (Figure 4); each of these bands consists of a compact cluster of isozymes. The B and D band isozymes are present in most tissues with some variability in distribution. The C band is seen only in placental tissue and is the heterodimer made up of B and D type subunits; it is not known why the C band is not formed in other tissues. The A band isozymes are considered to be derivative products of the B band isozymes since they are observed only in tissues containing B bands and desialidation reduces their

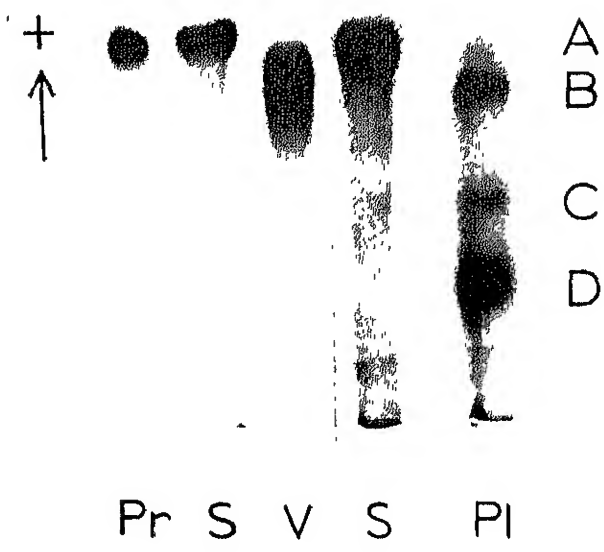

Figure 4. Characterization of acid phosphatase in prostatic tissue (PR), semen (S), and vaginal fluids (V) relative to the acid phosphatase in placenta (Pl). The starch gel electrophoretic system of Beckman and Beckman (1976) was used. (Reprinted from Sensabaugh 1982).

mobility to the B band position. Figure 4 shows that prostatic tissue gives a strong A and a weak B band, semen gives only an A band, and vaginal fluids give a broad band centering in the B band area.

Rare genetic variants found in surveys of placentas have allowed the definition of two gene loci, ACP_2 and

ACP_3. These loci code for the subunits of the placental D and B band enzymes, respectively (Beckman *et al.* 1970; Swallow and Harris 1972; Harris and Hopkinson 1976). (The ACP_1 locus encodes the low molecular weight acid phosphatase.) The B band mobility of the vaginal enzyme and the desialidated seminal enzyme suggests the possibility of ACP_3 locus origin. Immunological evidence supporting this notion is illustrated in Figure 5. The experiment indicates that seminal acid phosphatase and placental B band acid phosphatase are immunologically crossreactive; crossreactivity would not be expected if they were products of different loci.

Characterization of the high molecular weight enzymes using the low pH electrophoretic system of Lam *et al.* (1973) provides additional insights into acid phosphatase relationships. In this system, the acid phosphatase isozymes migrate cathodically yielding four major bands—1, 2, 3 and 4—in order of increasing cathodal migration (Figure 6); these bands, however, do not correspond one-to-one to the four bands seen in the Beckman system. Most tissues contain bands 1 and

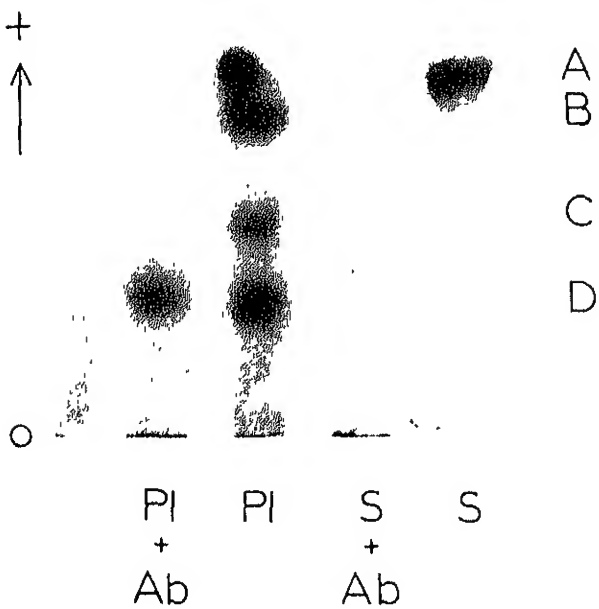

Figure 5. Demonstration of immunological cross-reactivity between seminal acid phosphatase and the ACP_3 gene product in placenta. Mixtures of enzyme and anti-seminal acid phosphatase antibody (Ab) were incubated overnight, centrifuged to remove immune precipitate, and analyzed by starch gel electrophoresis as in Figure 4. Control enzyme samples were incubated with nonimmune serum in parallel with the specific antibody treated samples. Reaction with antibody is indicated by removal of isozyme bands. Loss of the A and B bands affirms their relationship. Loss of the C band is consistent with the genetic evidence that it is a hybrid dimer containing one subunit from the ACP_1 locus and one subunit from the ACP_2 locus. (Reprinted from Sensabaugh 1982).

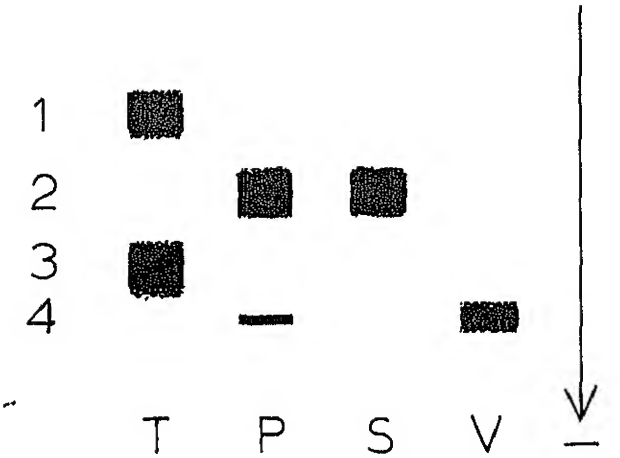

Figure 6. Schematic representation of acid phosphatase band mobilities in the electrophoretic system of Lam *et al.* (1973). Separation is on acrylamide gels in a pH 4.0 buffer system. Shown is a typical tissue pattern (T) and patterns from prostatic tissue (P), semen (S) and vaginal fluids (V).

3; based on charge considerations, these presumably correspond to the B and D bands respectively, in the Beckman system. Band 2 is the major component of semen and prostatic tissue; it is seen as a trace band in a few other tissues. Prostatic tissue also contains a small amount of band 4 isozyme, as do several other tissues including leukocytes, kidney, lung, and bone. The vaginal fluid acid phosphatase has band 4 mobility (Inman 1978). These observations suggest that bands 2 and 4 correspond to Beckman bands A and B, respectively. If this is so, then two Lam bands, 1 and 4, have B band mobility in the Beckman system; this in turn would suggest that B band isozymes might be encoded at two genetic loci, the ACP_3 locus and a locus not yet defined.

The four Lam band isozymes have been isolated by chromatography and partially characterized (Li *et al.* 1970; Yam 1974). Some differences are seen among them in substrate reactivity profiles but qualitatively they fit the basic profile indicated in Table 1. The band 2 and band 4 isozymes from prostate tissue and leukocytes are immunologically crossreactive but neither the band 1 nor the band 3 isozymes react with the test antisera (Lam *et al.* 1980). Moreover, desialidation of band 2 isozyme by neuraminidase treatment shifts its mobility to the band 4 position. Thus there is good evidence that the band 2 and band 4 isozymes are structurally related and that both are distinct from the band 1 and band 3 isozymes.

There is additional immunological evidence that tissues outside the urogenital tract contain acid phosphatases crossreacting with the prostatic enzyme. Foti

et al. (1977) demonstrated low levels of immunoreactive enzyme in many tissues using a radioimmunoassay technique. Sensitive immunohistological tests have demonstrated immunoreactive material in several tissues, notably in kidney and in leukocytes (Li *et al.* 1980; Shaw *et al.* 1981, 1982); these findings are in accord with the isozyme studies described above. Finally, Wojcieszyn *et al.* (1979) have reported the isolation of an acid phosphatase from female urine, presumably of kidney origin, which is enzymatically and immunologically identical to the prostatic acid phosphatase.

Taken together, the preceding provides evidence that prostatic acid phosphatase is neither biochemically nor immunologically nor genetically unique. The enzyme appears to be encoded at the ACP_3 locus which is expressed in a variety of tissues in addition to the prostate. What, then, is to be made of previous assertions of prostatic specificity (Shulman *et al.* 1964; Ablin *et al.* 1970; Vihko *et al.* 1978; Chu *et al.* 1978)? Most of these assertions have been based on immunological studies in which the immunological techniques have not been sensitive enough to detect the low enzyme levels in nonprostatic tissues. In the tissue survey of Foti *et al.* (1977), for example, the highest level of immunoreactive protein found outside the prostate was in pancreatic tissue and that was 1/900 the prostate level; such low levels would be very easy to miss.

It should be noted in conclusion that although qualitative specificity is lacking, the great difference in activity levels provides a kind of specificity. Foti's tissue survey showed the activity level in prostatic tissue to be 250 times higher than in any other tissue. As will be described in greater detail in the next section, the difference in activity levels between semen and vaginal fluids is on the order of 900. Thus, the amount of acid phosphatase in semen and in prostatic tissue far exceeds amounts found anywhere else in the body and if one sets an appropriately high activity detection threshold, only prostatic tissue and semen have enough activity to register.

DETERMINANTS OF ACID PHOSPHATASE LEVELS IN EVIDENCE MATERIALS

Basic Considerations

The acid phosphatase test has been employed both as a qualitative test and as a quantitative test. As a qualitative test, however, it is simply a quantitative test with a threshold; reactions exceeding the threshold are scored as positive and reactions below the threshold are scored as negative. Therefore, whether the test is to be used qualitatively or quantitatively, it is instructive to look quantitatively at the factors that determine acid phosphatase activity in stain and swab evidence materials.

Let us begin by taking an abstract look at the fate of a marker in dry stain material. A stain has a "life history"; it progresses from (a) the original liquid state to (b) the dry stain state to (c) the liquid state of the stain extract. The fate of a marker can be represented in terms of this life history thusly:

$$M_o \rightarrow M_d \rightarrow M_t \rightarrow M_e$$

where M_o is the amount of marker initially present in the liquid semen sample,
M_d is the amount of marker present in the initial dry state,
M_t is the amount of marker present in the dry stain at the time of extraction, and
M_e is the amount of marker detectable in the stain extract.

This life history scheme shows the three stages during which marker activity can potentially be lost: (a) as the liquid sample drys, (b) during the residence time in the dry state, and (c) in the extraction process. The amount of activity lost during each of these stages depends on definable determinant variables which are listed in Table 3. The amount of marker recovered in a stain extract depends on the amount of marker initially present, M_o, and the loss variables indicated in the table. Thus, given M_o and values for the loss variables, it is possible to predict M_e with reasonable accuracy.

Table 3. VARIABLES DETERMINING MARKER LOSS IN SEMEN STAIN MATERIAL

A.	Loss during the drying of the liquid sample
	1. Rate of marker loss in liquid semen (k_s)
	2. Time of drying (t_s)
	Amount lost $(M_o - M_d) = k_s t_s$
B.	Loss in the dry stain state
	3. Loss rate in dry state (k_d)
	4. Time in dry state (t_d)
	Amount lost $(M_d - M_t) = k_d t_d$
C	Loss during extraction and in the extract
	5 Extraction efficiency (x)
	6. Rate of marker loss in the extract (k_e)
	7. Time between extraction and analysis (t_e)
	Amount lost $(M_t - M_e) = xM_t + k_e t_e$

The situation with markers on vaginal swab evidence materials can be described in similar fashion. The life history of the sample begins with (a) the deposition of semen in the vagina, followed by (b) the postcoital

me on the swab, and concludes with (d) the extraction nd analysis of the swab. The fate of a marker can be epresented as before:

$$M_o \rightarrow M_c \rightarrow M_t \rightarrow M_e$$

where M_o is the total amount of the marker present in the vaginal fluids immediately after coitus, i.e., the sum of the semen contribution, M_s, and the vaginal contribution, M_v,

M_c is the amount of marker collected on the swab, including the contributions from the semen, M_s, and vaginal fluids, M_v,

M_t is the amount of marker surviving storage on the swab, and

M_e is the amount of marker detected in the swab extract.

This life history scheme shows that activity can be lost (a) in the vagina post coitus, (b) on the swab during storage, and (c) in the extract. The variables determining loss at each stage are indicated in Table 4; these variables are analogous to the variables determining loss in the stain situation. If the swab is dried following collection, the swab can be equated with the stain situation described above and the swab loss term can be factored into two terms, a loss-during-drying term and a loss-while-dry term. Whether or not the swab is dried, the swab life history scheme leads to the same conclusion as the stain life history scheme: the amount of marker detected in a swab extract is determined by the amount of marker initially present, M_o, and the values of the several loss variables.

Table 4. VARIABLES DETERMINING MARKER LOSS IN SWAB MATERIAL

A. Loss in the vagina post coitus
   1. Rate of marker in the vagina (k_v)
   2. Post coital interval prior to swab collection (t_v)

   Amount lost $(M_o - M_c) = k_v t_v$

B. Loss on the liquid state swab
   3. Rate of marker loss in liquid state swab (k_l)
   4. Time in liquid state on swab (t_l)

   Loss on the dry state swab (if the swab is dried)
   5. Rate of marker loss in dry state swab (k_d)
   6. Time in dry state on swab (t_d)

   Amount lost $(M_c - M_t) = k_l t_l + k_d t_d$

C. Loss during extraction and in the extract
   5. Extraction efficiency (x)
   6. Rate of marker loss in extract (k_e)
   7. Time between extraction and analysis (t_e)

   Amount lost $(M_t - M_e) = xM_t + k_e t_e$

ing the fate of markers in semen evidence material. It is recognized that given the vagaries of the real world, the loss variables can not be given precise values However, they can be defined within limits and these limits in turn set the boundaries on what is possible in the real world The following sections review what is known of the determinant variables affecting acid phosphatase levels in semen evidence materials Four areas are covered· (a) levels in semen, (b) levels in semen-free vaginal fluids, (c) rates of decline in the vagina post coitus, and (d) rates of loss in dry and liquid material under various conditions of storage. Unless otherwise attributed, the information to be presented is the result of collaborative studies with E T. Blake and J Bashinski.

Levels in Semen

Acid phosphatase activity levels in semen have been determined using a variety of substrates and assay conditions. As a result, the values reported vary from study to study. The value distributions, however, show similar features. The distributions span about two orders of magnitude and have an extended tail on the side of the higher values; a typical distribution is shown in Figure 7. Distributions such as these are often better described statistically by log-normal distribution parameters than by normal distribution parameters and this has been found to be the case with the semen acid phosphatase distributions. In other words, the logarithms of acid phosphatase values are better characterized by standard normal distribution statistics (e.g., means and standard deviations) than are the acid phosphatase values themselves. Of particular importance, the log-normal characterization gives an accurate picture of the low end of the acid phosphatase distribution; assumption of a normal distribution predicts that some 5 percent of semen samples have no acid phosphatase activity and this is simply not the case.

Figure 7 shows the distribution of acid phosphatase levels in semen samples from 255 individuals as determined by assay at pH 5.5 and 25 °C with p-nitrophenyl phosphate (pNPP) as the substrate. This assay has been used for much of the work noted in this section and will be referred to as the pNPP assay; details of the assay are described in a workshop presentation. The values found range from 14.5 to 1800 units/mL with a mean of 302 units/mL. The log-normal distribution mean corresponds to 235 units/mL and the plus and minus two standard deviation range is 53 to 1050 units/mL; these limit values bracket 95 percent of the

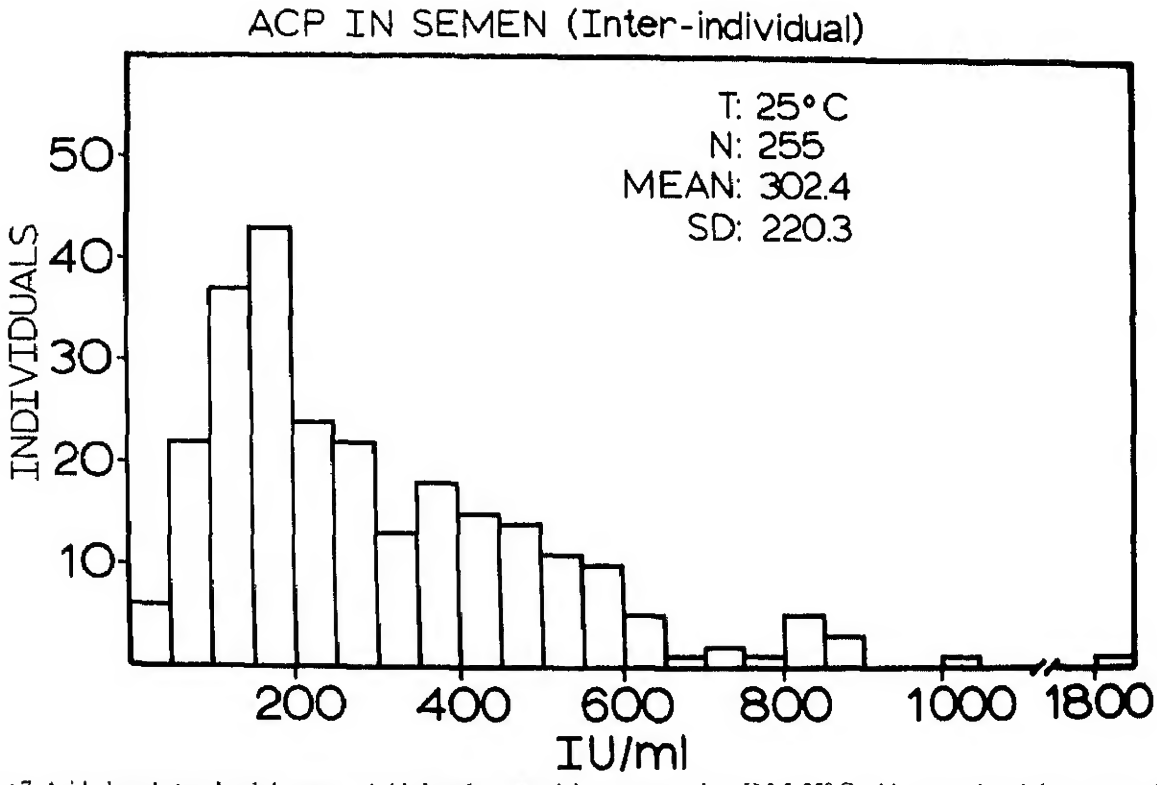

Figure 7. Acid phosphatase levels in semen. Acid phosphatase activity was assayed at pH 5.5, 25° C, with p-nitrophenyl phosphate as the substrate. A unit of acitivty is defined as the amount of enzyme catayzing and hydrolysis of 1 μ mole substrate per minute under the assay conditions. The assay procedure is described by Sensabaugh (1979) and in a workshop presentation at this meeting. (Figure courtesy E. Blake).

observed distribution. A similar range of values are reported by Brown *et al.* (1985) in their survey of 191 semen samples; using somewhat different assay conditions than those described here (37 °C instead of 25 °C), they found levels ranging from 10 to 1400 units/mL.

Information regarding the sources of the variation in enzyme levels is meager. There is clearly both variation between individuals and variation within individuals over time; however, the proportions of each to the total population variation cannot be gauged. Both inter-and intra-individual variations might be accounted for in part by variation in hormone levels; Engberg *et al.* (1947) showed a positive correlation between seminal acid phosphatase levels and circulating androgen levels. Hormonal variation possibly accounts also for age-related differences; the level of acid phosphatase in prostatic secretions is at maximum in males in their 20s and declines gradually thereafter (Kirk 1948; Kirk *et al.* 1952). Frequency of ejaculation, in contrast, has little effect on the acid phosphatase concentration in semen; however, semen volume decreases with shortened ejaculation intervals and, accordingly, total semen acid phosphatase is diminished (Eliasson 1965). It is clear that more could be learned about the determinants of acid phosphatase levels in semen. In particular, it would be of interest to know whether there are any conditions in which acid phosphatase is greatly reduced or is absent in semen.

In sum, we have a good picture of the quantitative distribution of acid phosphatase levels in semen even though we lack full understanding of the factors that determine inter- and intra-individual variations in levels. The information in hand shows that acid phosphatase levels fall in a broad but statistically definable distribution. It is thus possible to use acid phosphatase as a quantitative indicator for estimating amounts of semen present in evidence material; this application will be described in greater detail below.

Levels in Semen-Free Vaginal Fluids

Acid phosphatase levels in vaginal fluids have been determined in a number of studies (Sensabaugh 1979 and references therein; Allard and Davies 1979). As with semen, different substrates and assay conditions have been used and the reported values are not directly comparable. However, as with semen, the distributions of the data are comparable and analysis of these distributions shows that acid phosphatase levels in

vaginal fluids are log-normally distributed (Sensabaugh 1979). The delineation of the distribution makes possible statistical characterization of endogenous vaginal acid phosphatase levels; in particular, it allows the differentiation of endogenous and significantly elevated levels in vaginal fluids to be based on a sound statistical standard.

Measurement of acid phosphatase levels in neat vaginal fluids using the pNPP assay yields values ranging from 0.54 to 1.45 units/mL with a raw data mean of 0.34 unit/mL. Thus, semen and vaginal fluid levels differ by a factor of about 1000. Swabs used for vaginal fluid collection take up approximately 0.1 mL and hence would be expected to contain about 0.034 unit. This corresponds well to values obtained in published and unpublished studies of vaginal swab levels; data means from the swab studies (after adjustment to the same unit definition) range from 0.015 to 0.035 units per swab. This correspondence indicates that little activity is lost on swabs.

The statistical analysis of endogenous vaginal acid phosphatase distributions indicates that 99 percent of endogenous values fall below a value that is 6.6 times the raw data mean (Sensabaugh 1979). Given the mid-value of the swab data means, 0.025 unit/swab, the 99 percent threshold value is 0.165 unit/swab. The 99.9 percent threshold value is 14.2 times the data mean: 0.355 unit/swab. These threshold values are approximately two orders of magnitude lower than what would be found on an average swab containing neat semen (30 units/swab given the 302 units/mL semen average value); in other words, the significance range factor of the acid phosphatase test is about 100.

This approach can be used to set significance thresholds for any defined unit system. For example, the assay with thymolphthalein monophosphate as a substrate is often reported in units defined as μ moles substrate hydrolyzed per minute per liter swab extract. Findley (1977) reported a mean vaginal acid phosphatase level of 25.75 TMP units/L. Starting with this value, the 99 percent and 99.9 percent threshold values are, respectively, 170.1 and 365 TMP units/L.

Although the variation in vaginal enzyme levels is well characterized statistically, little is known of its biological basis. The distributions of values for individual women are about ¼ to ½ as wide as the population distribution; thus variation within individuals is considerable. Time of cycle does not appear to contribute significantly to this variation (Lantz and Eisenberg 1978; Sensabaugh 1979). There is some indication that levels decline with age (Maners *et al.* 1982); levels in women over 50 were found to be about 1/3 of levels in younger women. Nothing specifically is known of the effects of pregnancy, parity, frequency of intercourse, or vaginal infection, although there is no indication that any of these factors boost levels above the defined normal vaginal range.

The effect of death on vaginal acid phosphatase levels has not been well characterized. It might be anticipated, however, that acid phosphatase activity levels would increase. Postmortem tissue breakdown results in the release of many enzymes, including acid phosphatase, into the extracellular space. In addition, outgrowth of the resident microbial flora may contribute acid phosphatase enzymes to the vaginal vault fluids. Until more is learned about postmortem effects, acid phosphatase tests on postmortem swabs should not be interpreted by the same standard as tests on swabs from live victims.

In sum, the existing empirical and statistical data base is sufficient to allow a meaningful interpretation of acid phosphatase levels in vaginal swab evidence from live victims. Interpretation of postmortem swabs is more problematic and more needs to be learned in this area.

Rate of Postcoital Decline in the Vagina

The decline of acid phosphatase activity in the vagina after sexual intercourse is the sum result of several processes including (a) dilution by vaginal fluids, (b) drainage from the vaginal vault, and (c) degradation by vaginal and/or seminal hydrolases. Dilution and drainage are generally considered the dominant mechanisms of loss, at least in the earlier stages, although the relative contribution of these processes to the rate of postcoital decline is not well characterized. We do have, however, a reasonably good picture of the overall rate of postcoital decline from the studies of Sensabaugh (1979) and of Rutter *et al.* (1980). The data from these two studies are compatible and have been combined for the analysis presented here; the combined data set includes over 400 data points.

The pattern of the postcoital decline is illustrated in Figure 8; the normal ranges for semen and for semen-free vaginal fluids are also shown. The acid phosphatase activity coordinate is scaled relative to the mean vaginal acid phosphatase level which is defined as having a standardized ACP value of one; given the mid-value vaginal mean used above (0.025 pNPP unit/swab), standardized ACP values of 10, 100, and 1000, correspond, respectively, to pNPP unit/swab values of 0.25, 2.5, and 25. The observed values in each postcoital interval span two or more orders of magnitude and are log-normally distributed. The dashed lines represent the plus and minus two standard deviation limits in each interval; more than 95 percent of postcoital values fall within the envelope thus defined.

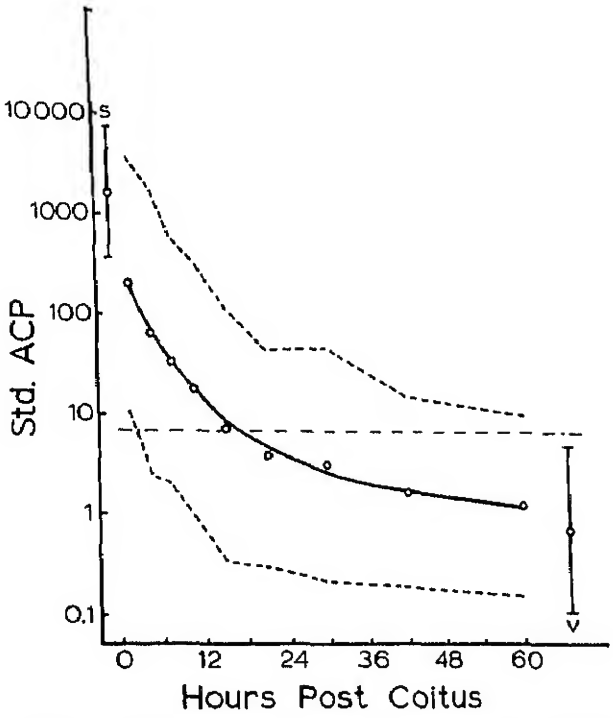

Figure 8 Post coital decline of acid phosphatase levels in vaginal fluids. The solid line indicates the decline in mean values in the following post coital intervals (in hours). 0 -3, 3 -6, 6 -9, 9 -12, 18 -24, 24 -36, 36 -48 and 48 -72 The dashed lines indicate the plus and minus two standard deviation ranges of the distribution of values in each interval, 95 percent of the values in each interval fall between the lines The horizontal line (— —) indicates the 99 percent threshold of the normal vaginal range. The normal ranges (+/−2 standard deviations) of acid phosphatase activity in semen (S) and vaginal fluids (V) are indicated Standardized acid phosphatase values are defined in the text The figure is based on a combined analysis of data from Sensabaugh (1979) and Putter *et al* (1980), over 400 data points were included in the analysis

It is clear that there is a regular pattern of acid phosphatase activity decline. The loss of activity is approximately log-linear (first order) through the first 10 hours with an activity half-life of about 2.6 hours. The rate of loss then becomes more gradual and by 60 hours, the distribution of values is close to that found in semen-free vaginal fluids. The overall rate of decline in the vagina is much more rapid than the rate of activity loss in test-tube mixtures of semen and vaginal fluids (next section) which is consistent with the idea that the dominant mechanisms of loss are dilution and drainage rather than chemical inactivation.

The figure illustrates two significant points about the acid phosphatase test. First, postcoital acid phosphatase values begin to fall in the endogenous range within a few hours after intercourse and do so with increasing frequency as the postcoital interval increases. These values are "false negatives." The percentage of false negatives as a function of postcoital interval is given for two data sets in Table 5. Both data sets show a significant proportion of false negatives even in the first six hours postcoitus; the proportion of false negatives begins to exceed 50 percent after about 15 hours. The lesson here is that a negative acid phosphatase test does not indicate the absence of an earlier ejaculation in the vagina.

Table 5. "FALSE NEGATIVE" ACID PHOSPHATASE TESTS

Postcoital Interval (hrs)	*Percent False Negatives (99 percent threshold)*	
	Sensabaugh[a]	*Allard and Davies*[b]
0 -6	8	21
6 -12	33	41
12 -18	53	55
18 -24	66	69
24 -36	76	86

[a]Sensabaugh (1979) predicted values.

[b]Allard and Davies (1979) have set their significance threshold at 20 Sigma units/swab Analysis of their data distribution yields a 99 percent threshold at about 23 Sigma units/swab.

The second point is that the acid phosphatase test cannot yield precise estimates of postcoital intervals. This point follows from the broad distribution of values over successive postcoital intervals and is illustrated by the data in Table 6. For example, standardized values in the range 64 -128 (corresponding to 1.6 to 3.2 pNPP units/swab) are observed with moderate frequencies in each of the first four 3-hour interval blocks; given a value in this range, one could not, with any confidence, assign an exact interval or even a

Table 6. ESTIMATION OF POSTCOITAL INTERVAL: FREQUENCY OF OBSERVING AN ACID PHOSPHATASE VALUE IN DIFFERENT POSTCOITAL INTERVALS

Postcoital Interval	*Observed ACP Value (Standard Units)*		
	256 +	*64 -128*	*16 -32*
0 -3	60	.33	.06
3 -6	.25	.25	.17
6 -9	.10	.19	.19
9 -12	03	.12	.18
12 -18	.01	.06	.15
18 -24	—	.03	10
24 -36	—	.01	.07
36 -48	—	—	.04
48 -72	—	—	.02
72 +	—	—	01

narrow interval range. Lower standardized ACP values are even more evenly distributed and over longer interval periods. About the best that one can do is state the interval limit beyond which any particular value would be unlikely. Thus, for example, standardized ACP values exceeding 256 (6.4 pNPP units/swab) are very unlikely after 12 hours and standardized ACP values in the 64–128 rnge are unlikely after 24 hours

Rates of Loss in Stains and Swabs

The principal environmental determinants of marker survival in evidence material are temperature, degree of wetness, and the surrounding chemical environment. Of these, the degree of wetness is the most critical since the nature and rates of deterioration processes differ markedly in wet and dry materials (Sensabaugh 1983). Wet-state deterioration is dominated by enzyme-mediated degradation processes. In dried materials, slower processes, probably involving dehydration condensations and free radical reactions, are dominant; enzyme-mediated processes are not significant.

It should be noted that dry materials are not completely dry; proteins bind water tenaciously and even material that appears quite dry can contain some 10 percent of its weight in water. The amount of water bound is in equilibrium with the partial pressure of water vapor in the surrounding atmosphere, i.e., the relative humidity. The boundary between wet- and dry-state processes occurs at water content levels equilibrating at about 75 percent relative humidity. Thus, in humid environments, "dry" stains or swabs may not really be very dry and may be subject to wet-state deterioration processes.

The rates of acid phosphatase loss in dried semen, liquid semen, and semen-vaginal fluid mixtures are shown in Table 7. The data show that acid phosphatase is quite stable under the conditions tested; the shortest half-life listed is about one month and the longest is on the order of years. To put these times into context, almost six half-lives must pass to reach a 98 percent loss of activity. Thus, evidence samples bracketed by the conditions indicated in the table would not be expected to loose much acid phosphatase activity in real time.

Table 7. ACID PHOSPHATASE: RATES OF LOSS

	Half-Life Times			
Semen State	*-20 °C*	*4 °C*	*20 °C*	*37 °C*
Air dried stain	no loss 1 y	5 y (est)	1–1.5 y	4–6 mo
Liquid	10–20 y	—	4.6 mo	2–3 mo
Liquid—mixed with vaginal fluids	>6 mo*	>6 mo*	2–3 mo*	1 mo

Times: y–years; mo–months; est–estimate. All data from Blake (unpublished) except those with an asterisk (*) which are from Standefer and Street (1977).

The data in the table make, in addition, two points pertaining to evidence stability and preservation. First, at each temperature, acid phosphatase activity is more stable in the dried material than in the liquid material. Similarly, the activity is more stable in neat liquid semen than in the semen-vaginal fluid mixtures; the difference indicates that vaginal fluids contain factors contributing to the deterioration processes. Second, in any given state, enzyme activity is more stable at lower temperatures; this, of course, is to be expected. These two points provide guidance for the preservation of evidence materials; the greatest stability is to be found in material preserved cold and dry.

The stability of acid phosphatase activity in postmortem semen-vaginal fluid, semen-oral fluid, and semen-rectal fluid mixtures has been characterized by Standefer and Street (1977). Enzyme activity is less stable in oral and rectal fluids than in vaginal fluids; activities in the former are substantially lost after 15 days at 25 °C. Although the fluid samples were collected postmortem, the data provide reasonable estimates of premortem loss rates in these fluids in the absence of dilution and drainage effects. An additional point regarding acid phosphatase stability in solution is that total protein concentration is important (Lantz and Eisenberg 1978); semen diluted 1/100 in a solution containing 5 percent albumin lost acid phosphatase activity much more slowly than semen similarly diluted into a protein-free solution. Interestingly, we have found acid phosphatase in dried stains is similarly stabilized by surrounding protein although the effects are not as dramatic as those seen in solution samples.

In sum, acid phosphatase is quite stable under most conditions encountered by evidence samples. Moreover, under appropriate storage conditions, little, if any, activity should be lost, even if the storage is for months or years.

Acid Phosphatase as a Quantitative Indicator of Semen

The relative stability of acid phosphatase advertises its great value as a marker for semen. Following through on the life history schemes outlined at the beginning of the section, it can be seen that activity survives in stain material with relatively little loss and that the major determinant of activity level in swab

evidence is the loss from the vagina postcoitus; in the latter case, the major losses almost certainly reflect semen dilution in and drainage from the vagina.

These observations lead to the suggestion that acid phosphatase activity be used as a quantitative indicator of the amount of semen present in stain and swab extracts. An approach to semen content estimation and its application to case samples has been devised by Blake and is detailed in a workshop presentation at this meeting. The essence of this approach is to base the estimate on a high assumed initial semen acid phosphatase value (600 pNPP units/mL, the 90th percentile value); this leads to a conservative underestimate of the true semen content. To illustrate, assume a swab extract contains 3 pNPP units/mL acid phosphatase activity. Using the 600 units/mL estimator value, the swab extract would be estimated to contain semen at a 1/200 dilution. If the seminal ejaculate contained initially 300 units/mL, the true semen dilution would be 1/100; in other words, the extract would, in fact, contain twice as much semen as estimated. Since 90 percent of semen values are lower than the 600 units/mL index value, the semen content of the extracts would be underestimated 90 percent of the time. If the initial semen sample by chance happened to be in the 10 percent with an acid phosphatase level exceeding 600 units/mL, the semen content estimate would be low but with rare exception by no more than a factor of two, i.e., one serial dilution factor.

The estimation of semen content in swab and stain extracts is of value in corroborating impressions of semen content from sperm searches. More significantly, this information can be used to assess the potential for success in subsequent genetic testing and as accessory information in the interpretation of genetic testing results. Thus, this quantitative application adds a new dimension to the usefulness of the acid phosphatase test.

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DISCUSSION

Blake: What do the 256 units refer to?

Sensabaugh: Those are standardized units, not international units. I found that when I started doing work with quantitative acid phosphatase, a number of years ago, everyone used a different assay system and hence reported the results in terms of different units. The only way I could make sense of it was to try and standardize in some way. So, I developed a trivial arithmatic normalization procedure which would allow data from different workers to be compared. Those 256 units that I made reference to come from using that normalization.

Question: How stable is acid phosphatase?

Sensabaugh: Very simply, it is a very stable enzyme.

IMMUNOLOGICAL METHODS FOR SEMINAL FLUID IDENTIFICATION

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HISTORICAL PERSPECTIVE

The ability to conclusively identify semen stains by detection of a component unique to this body fluid has been the product of technologies and information developed from unrelated lines of scientific inquiry. For decades, forensic scientists had recognized the need for a seminal fluid diagnostic test which did not rely upon sperm cell presence. Only within the last five years has this goal been achieved through a convergence of studies in both forensic and clinical sciences.

Early in the 1970's, clinical scientists, evaluating potential immunological approaches toward fertility control, were studying the antigenic profile of human seminal plasma. Based upon immunoelectrophoretic separations and characterization, Li and Shulman (1971) resolved seminal plasma into several groups. One antigenic component termed E was chosen for further study (Li and Beling 1973) because it appeared to be a major antigenic species in seminal fluid. Biochemical dissection of E revealed that it was primarily one component, E_1, with a smaller quantity of E_2 being present. Further characterization of E_1 indicated that this component was a protein of molecular weight 31,000 which was devoid of carbohydrate or lipid residues. Because an antiserum raised against human prostatic tissue failed to precipitate a molecular species from seminal plasma identical to E_1, it was concluded by these investigators that E_1 was not of prostatic origin. Subsequent studies by another (Sensabaugh 1978) suggest that the genesis of E_1 is, in fact, the prostate gland, and the inability of Li and Beling to demonstrate its tissue origin was due perhaps to insensitivity of their detection system.

Several early reports in the forensic literature described a seminal fluid-specific protein (gamma-seminoprotein, Koyanagi *et al.* 1972; Hara *et al.* 1972). However, Sensabaugh was the first in the forensic science arena to thoroughly and systematically characterize the proteins present in seminal fluid in order to identify a molecular species absent from other major body fluids (Sensabaugh 1978). Electrophoresis of semen on polyacrylamide gels containing sodium dodecyl sulfate revealed a protein of molecular weight 30,000 which was absent from blood plasma, vaginal secretions and milk. This component was termed p30. Extensive purification of p30 from semen by ion-exchange chromatography and gel filtration confirmed the molecular weight and suggested that the protein exists as a monomer in its native state in seminal fluid. Antisera prepared against the E_1 protein isolated by Li and Beling were cross-reactive with p30 suggesting that some immunologic determinants were shared by these species. Further immunologic characterization of p30 indicated that the protein was absent from extracts of the testicle, seminal vesicle and vas deferens. In contrast to the study by Li and Beling, antisera to p30 precipitated an antigen from an extract of prostatic tissue.

Within the time frame during which Sensabaugh was conducting studies on p30, investigators in the field of tumor immunology were searching for blood-born antigenic markers which might enable early diagnosis of prostatic cancer. Wang *et al.* (1979) described the purification of an apparently tissue-specific glycoprotein from prostate gland. This protein was termed prostate antigen or PA. Biochemical characterization indicated a molecular weight for PA of 33,000 to 34,000 and an isoelectric pH of 6.9. Immunohistochemical staining studies indicated that the origin of PA is the epithelial lining of the prostate gland ductal elements (Nadji *et al.* 1981).

For patients with advanced prostatic cancer, PA is elaborated into peripheral blood in sufficient quantities to be detected by conventional methods such as rocket immunoelectrophoresis (Papsidero *et al.* 1980). Utilizing a sensitive enzyme-linked immunoassay, PA can be demonstrated in the peripheral blood of normal males, although the levels are approximately 10^6 times lower than that seen in seminal fluid (Kuriyama *et al.* 1980). The molecular size of PA in peripheral blood was found to be about 100,000, considerably larger than the semen-associated PA. Biochemical studies revealed that blood-born PA is associated noncovalently with a large carrier molecule (Wang *et al.* 1981). In a strict sense, PA is not unique solely to semen but is normally present in blood, albeit beneath the detection limit with the common methods used for its detection.

Summary of Biochemical Properties and Tissue Distribution of p30

Table 1 highlights the biochemical properties of p30 and PA. Both molecules are of similar molecular weights and both contain carbohydrate substituents on the protein core. Studies by Blake (personal communication) indicate that sialic acid contributes substantially to the electrical charge exhibited by p30 and that different degrees of sialidation are responsible for the observed differences in isoelectric pH.

Table 1. BIOCHEMICAL CHARACTERISTICS OF SEMEN-SPECIFIC PROTEINS

p30[a]		PA[b]
30,000	molecular weight	33,000–34,000
glycoprotein	composition	glycoprotein
6.5–8.0	isoelectric pH	6 9
yes	isomeric	yes

[a] Sensabaugh 1978 [b] Wang *et al* 1981

Since the available literature reports suggest that p30 and PA are identical biochemically (Wang *et al.* 1983), they will be taken as equivalent for the remainder of this work. The term p30 will be used generically since it is in common forensic parlance.

The tissue distributions of p30 are shown in Table 2. A considerable number of human tissues have been examined for the presence of p30, certainly all those commonly encountered in forensic situations It should be noted that p30 can be detected readily in seminal fluid as well as prostatic tissue and *in vitro* cultured prostatic malignancies. Peripheral blood occurs under both column headings since p30 may or may not be detected in this fluid depending upon the assay system employed.

Table 2. TISSUES AND BODY FLUIDS EXAMINED FOR THE OCCURRENCE OF p30[a]

Detected In	*Not Detected In*
Seminal Fluid	Peripheral Blood
Prostatic Tissue	Testis
Prostatic Carcinoma	Seminal Vesicle
— In Situ	Vas Deferens
— In Vitro	Erythrocytes
Peripheral Blood	Tears
Urine	Perspiration
	Saliva
	Milk
	Menstrual Blood
	Stomach Tissue
	Small/Large Intestine
	Pancreas
	Liver
	Kidney
	Breast
	Non-Prostatic Carcinomas
	Vaginal Fluid

[a] Compiled from Nadji *et al* (1981) and Sensabaugh (1978).

p30 CONTENT OF HUMAN BODY FLUIDS

The mean concentration of p30 in human semen has been reported to be as low as 800 μg/ml semen (Blake *et al.* 1982) and as high as 1900 μg/ml (Sensabaugh 1978). Other studies (Brown *et al.* 1985) have shown the mean value to be 1200 μg/ml. These dramatic differences are undoubtedly a reflection of the degree of purity of the p30 standards used to calibrate the assays in these studies. For example, for a single semen sample assayed against p30 standards of different specific activities (μg p30/mg total protein), the p30 concentration of the sample will apparently decrease as the specific activity of the standard increases. Thus, the forensic laboratory which performs quantitative p30 analyses for interpretative purposes must ascertain its own population distribution data based upon the purity of its p30 standards. For the p30 standards which yield a mean semen p30 concentration of 1200 μg/ml, the range of values observed has been from 300 μg/ml up to 4200 μg/ml. This range is based upon a sample population of more than 200 specimens.

P30 cannot be detected in the serum of normal males when sought by conventional immunoassay methods such as rocket immunoelectrophoresis or radial immunodiffusion. About 8 percent of males with far advanced prostatic cancer will exhibit serum p30 levels detectable by these assay methods (about 0.5μg p 30/ml/ Wang *et al.* 1981). Sera from normal males may contain p30 at levels as high as 0.0026 μg/ml serum. Such levels are detectable only by enzyme-linked immunoassay. Sera from females are devoid of p30.

Despite the fact that p30 is not exclusively confined to semen, the forensic serologist need not be concerned with the occurrence of this substance in any other human body fluid except perhaps in urine where it may appear by spillage from the prostate (Blake, personal communication).

IMMUNOLOGICAL METHODS FOR p30 DETECTION IN SEMEN STAINS

P30 possesses no known intrinsic activity which can be directly measured. Thus, all detection methods for this substance must exploit its antigenicity. Five methods will be described for p30 detection. Four are based upon immunodiffusion and the fifth is an

enzyme-linked immunoassay. All test procedures are described as they have been performed in our laboratory. With the exception of the enzyme-linked immunoassay, all detection methods utilize commercially available antisera to p30.

1. Double Immunodiffusion

A. *Mechanism*

The detection of an antigen by double diffusion in agar is based upon the principle that for many homologous antigen-antibody pairs there is an optimal ratio of their proportions at which the antigen-antibody immune complexes are too large to remain soluble. By permitting antigen and antibody to diffuse toward one another through a semisolid medium such as agar, an overlap of concentration gradients is formed. Although antigen and antibody interactions occur throughout the region of overlap, only within a narrow zone are the species present in the optimal proportions favorable for precipitation of the complex. If antigen and antibody are introduced to their respective wells in approximately equivalent concentrations, the precipitin line will develop midway between the wells of origin. If either component of the system is initially present in great excess relative to the other, precipitation will occur closer to the well of lower concentration.

B. *Application to Semen Stains*

Eight ml of 1 percent molten type I agarose in Hepes-buffered saline pH 7.5 (0.1M HEPES: 0.144 M NaCl) (HBS) is poured carefully onto clean 5 x 7.5 cm glass slides. Wells of 2.5 mm diameter are aspirated from the gel with centers at least 5 mm apart. Semen stain extracts should be tested neat and at a 10-fold dilution. Commercially available antisera to p30 can be used at a two-fold dilution. Opposing sets of wells are filled (approximately 10 µl) with extract and antisera, respectively, and the plate is placed in a humid chamber at room temperature for development.

After 24 hours, precipitin lines will be apparent between the wells when examined by indirect light against a dark background.

C. *Utility of Method*

This is perhaps the most basic immunologic test that can be performed to identify semen. The lower limit of sensitivity is on the order of 10 µg antigen protein/ml.

Although double diffusion is primarily a qualitative technique, it may be applied semiquantitatively. One may test serial dilutions of a semen stain extract in parallel with dilutions of a semen specimen of known p30 content. The last dilutions of extract and standard at which a precipitin line remains visible can be used to calculate approximately the relative concentration difference between extract and standard.

2. Counterimmunoelectrophoresis

A. *Mechanism*

Counterimmunoelectrophoresis (CIEP) is an electrophoretic variation of double immunodiffusion. With double immunodiffusion, antigen and antibody are permitted to commingle and form precipitates as a result of simple diffusion through an agar gel. CIEP accelerates this union by electrically driving the antigen toward the antibody. Antigen and antibody are placed in opposing wells in a gel composed of an agarose type which has a high electroendosmotic value. Typically the agarose gel is prepared in an alkaline buffer which will impart a net negative charge to the protein antigen and antibody molecules. Upon application of an electromotive force across the gel, the antigen molecules will be pulled toward the anode. Antibody molecules, which are weakly charged, will be swept toward the cathode by the electroendosmotic force within the gel. Union and precipitation of antigen and antibody occur between their respective wells within a very short time.

B. *Application to Semen Stains*

Three ml of 1 percent type III agarose (EEO = 0.25) are prepared in 50 mM barbital buffer at pH 8.6. After liquefaction of the agarose, the gel is formed on standard glass microscope slides. Wells of 2.5 mm diameter, 10 mm apart, are prepared to receive samples of 5 µl. Numerous pairs of opposing wells can be placed on one gel slide. Semen stain extracts should be tested neat and at a dilution of 1:10. These samples are placed in the wells of what will be the cathodic side of the gel (migration will be toward the anode). Anti-p30 antiserum, diluted no more than 1:2, is placed in each of the opposing wells. Wicks are attached (three layers of No. 3 Whatman filter paper) and 10 volts/cm are applied across the gel for 30 minutes. Precipitin lines may be visible without gel staining. If staining is desired, the gel may be washed, dried and stained as described later.

3. Radial Immunodiffusion

A. *Mechanism*

Radial immunodiffusion (RID) (Vaerman 1981) is an immunodiffusion technique based upon the principle that as an antigen diffuses through an agar gel containing uniformly dispersed homologous antib-

ody, the area of precipitation which forms is related quantitatively to the concentration of antigen present. When the diffusion of antigen is complete, and the endpoint has been reached, there is a simple linear relationship between the area of precipitin ring and the quantity of antigen initially introduced into the well. The relationship between precipitin area and antigen concentration for systems which have not reached the endpoint is governed by more complex mathematical functions (Vaerman 1981) which need not be introduced. The time required to reach the endpoint of diffusion varies as a function of the size of the antigen. For substances of molecular weight up to about 100,000 (which includes p30), the minimum diffusion time is 24 hours. In practice, this time should be empirically determined for each antigen-antibody system.

B. *Application to Semen Identification*

1. *Determination of the Optimal Level of Anti-p30 in the Gel*

The initial step in establishing an RID assay for p30 in semen stain extracts is the empirical determination of the amount of homologous antibody to incorporate into the agarose gel. This is an important assay consideration. If the concentration of anti-p30 antiserum in the gel is too high, all p30 will be precipitated within a small area surrounding the well, with a commensurate loss of assay sensitivity. Conversely, if insufficient anti-p30 is present in the gel, the precipitin rings will grow too large and faint to be visualized.

A series of three agarose gels is prepared each containing a different volume of anti-p30 antiserum. For commercially available antisera to p30, it is suggested that final antiserum concentrations of 2.0 percent, 1.5 percent, and 1.0 percent (v/v) be tested. Each gel is tested with the same series of p30 standards ranging as high as 40 μg p30/ml. After the diffusion is complete, a decision can be made as to the antiserum concentration which is optimal for the sensitivity desired (see interpretation).

2. *Routine Evaluation of Semen Stains*

Six ml of molten type I agarose in HBS is prepared for each RID plate. After solution of the agarose is effected at 100° C, the tube is placed in a water bath set at 50°C. It is essential that adequate time be allowed for the agarose to temperature equilibrate at 50°C. Addition of antiserum prematurely may result in rapid and total loss of anti-p30 activity due to thermal denaturation of the antibody protein. Ten minutes at 50°C should suffice to achieve temperature equilibrium.

Anti-p30 antiserum is added to the agarose in a volume previously determined to be appropriate to the level of sensitivity desired. The antiserum is mixed into the agarose by gently swirling the tube contents. The formation of air bubbles is to be avoided. The mixture is poured onto 5 cm × 7.5 cm glass slides and uniformly spread over the slide surface. After gelation, wells spaced 1 5 to 2 cm apart are prepared by aspiration with a 2.5 mm punch. Semen stain extracts should be tested neat and at a dilution of 1:10. Liquid semen samples must be diluted at least 50-fold.

Assay plates for semen stain extracts should be accompanied by at least four concentrations of p30 standards (e.g., 40, 20, 10 and 5 μg/ml). Five μl aliquots of extract or standard are introduced into each well with a micropipettor or microsyringe. Careful quantitative technique in filling wells is essential. The plate is placed in a humid chamber at room temperature for at least 24 hours.

3. *Washing, Drying and Staining*

In the author's experience, precipitates of p30 and its homologous antibody are not readily visible in the wet gel; thus, drying and staining are necessary before measurements can be taken. First, the unprecipitated protein must be removed by washing the gel. Cover the gel surface with a single layer of moist filter paper (e.g., Whatman No. 1), being careful to avoid trapping air bubbles. Six thicknesses of Whatman No. 3 filter paper are placed over the covered gel followed by a glass plate. A weight of about 200 g is put on the entire sandwich. Within 20 minutes most of the liquid within the gel will have been absorbed by the filter paper pad. Place the gel plate in a suitable container and cover with 1M NaCl. Permit the gel to soak overnight and then wash once with distilled water for 30 minutes. During the wash procedure the gel is apt to loosen from the glass plate, so care must be taken to avoid damage to the gel. Repress the gel with filter paper. Drying is effected by removing the paper and placing the plate into an oven set at between 37°C and 50°C. For staining, the plate is covered with a solution of 0.5 percent Coomassie Blue dissolved in a methanol:acetic acid:water mixture (9:2:9) which serves also as the destain solution. Staining time is about 5 minutes. Destain with several changes of solvent and rinse with water. The gel may be blotted dry with soft paper lab towels.

4. *Interpretation*

The stained gels are placed on a light box and the diameters of the precipitin rings measured to the near-

est 0.5 mm. Measure two perpendicular diameters for each ring to compensate for deviations from true circularity. The product of these two diameters is taken as the area of the ring. Since pi is a common factor to all rings, it need not be used in the calculation.

On graph paper, plot the areas of the precipitin rings versus their respective p30 concentrations. One may read the concentrations of p30 in the questioned specimens directly from this standard curve.

A common pitfall in RID interpretation is extrapolating the standard curve so as to accommodate a sample precipitin ring which is larger than that given by the highest concentration of standard. Under such circumstances, the specimen must be rerun at a greater dilution so that its ring falls within the standard curve range.

4. Rocket Immunoelectrophoresis

A. *Mechanism*

Rocket immunoelectrophoresis (RIEP) is also known as electroimmunoassay or electroimmunodiffusion (Laurell and McKay 1981). The principle of this quantitative immunoassay is that the area of the antigen-antibody precipitation is proportional to antigen concentration when the antigen is driven electrically into an agar gel containing nonmigrating homologous antibody. The precipitin patterns resemble spikes or "rockets." The rocket shape occurs because immune complex precipitation takes place at the lateral edges of the moving boundary of antigen molecules. As antigen concentration diminishes due to precipitation, the lateral margins of the antigen front begin to converge, ultimately forming the rocket tip. The distance of antigen migration is linearly related to antigen concentration for a given concentration of antibody.

B. *Application to Semen Identification*

1. *Determination of Optimal Level of Anti-p30 to Incorporate Into the Gel*

At a fixed concentration of anti-p30 antiserum, the heights of p30 rockets will be directly proportional to their p30 concentrations. In contradistinction, at a standard concentration of seminal fluid p30, the rocket height will be inversely related to anti-p30 antiserum concentration. Thus, one must empirically test several antiserum concentrations so as to achieve the desired sensitivity range for the assay. The following procedure is designed to permit quantification of p30 at a concentration not to exceed 40 μg/ml.

Three gels are prepared each containing a different concentration of anti-p30 antiserum. Generally, gels containing about 1 percent, 2 percent and 3 percent (v/v) anti-p30 will span the sensitivity range desired. Each gel is tested with the same seminal p30 concentrations. When the run is complete, one chooses for general use the antiserum concentration which has given the highest rockets which are visible for the p30 standard of greatest concentration.

2. *Quantification of p30 in Semen Stains*

A 5.6 ml portion of molten Type I agarose in 50 mM barbital buffer at pH 8.6 is prepared for each RIEP plate. The agarose must be equilibrated at 50° C prior to antiserum addition for reasons described earlier. Anti-p30 antiserum is added to the agarose in a volume previously determined to be optimal for rocket development. The agarose:antiserum mixture is poured onto a 5 cm × 7.5 cm glass plate and permitted to gel. A gel layer 0.15 cm thick will result. A series of 12-2.5 mm diameter wells are aspirated 1.0 cm from the bottom edge of the gel. Wells are spaced 0.5 cm apart, but no closer than 1.0 cm to the lateral edges. Each well will receive 5 μl of semen stain extract. Each semen stain extract is run neat and at a 1:10 dilution to ensure that at least one dilution will give a rocket height which lies within the standard curve range. It is essential that p30 standards be included on each gel. In the present case, p30 standards of 40, 20, 10 and 5 μg/ml would be used. The gel plates are placed on a plastic platen through which chilled water is circulated. Three Whatman No. 3 filter paper wicks are installed on each side of the gel and immersed into the tank buffer (50 mM Barbital, pH 8.6). Voltage is set at 7.5 v/cm and the run time is 4 hours. Migration is toward the anode.

3. *Drying and Staining*

The completed RIEP gel is dried and stained as described in the RID section. Washing is unnecessary since the incidental serum proteins (albumin, α and β globulins) migrate rapidly to the anodic end of the gel and into the wick during the four-hour run.

4. *Interpretation*

The stained gels are placed on a light box and the rocket heights measured to the nearest 0.5 mm. A standard curve is constructed by plotting height of the p30 standards versus p30 concentration in μg/ml. The p30 content of the semen stain extracts can be determined from the standard curve. Any rocket whose height exceeds that of the greatest p30 standard cannot be used for quantification. Since each extract is run at

two concentrations, generally one rocket of the pair will lie upon the standard curve.

The lower limit of sensitivity for RIEP is about 5 μg/ml. If increased sensitivity is desired, less anti-p30 antiserum is required in the gel. When conditions are adjusted so as to increase sensitivity, there will be a commensurate loss in the ability to measure higher levels of p30.

5. Enzyme-linked Immunoassay

A. *Mechanism*

All enzyme-linked immunoassays (ELISA) employ an enzyme-conjugated antibody as a reagent for the detection of homologous antigen. Although there are numerous technical variations of the ELISA, only one procedure will be described in this presentation. Additional information can be found in a recent review (Engvall and Ruoslahti 1979) of the technology. In this description we shall be dealing with a solid state ELISA. The technique incorporates the following steps and principles:

1—Specific antibody is noncovalently adsorbed to a solid phase such as the wells of a polystyrene microtiter plate or test tube. This is usually done at an alkaline pH (e.g., 9.6) which encourages adsorption of the antibody to the plastic.

2—Sample containing the antigen to be tested is added to the vessel. The immobilized antibody will bind antigen during the incubation period.

3—A second antibody is introduced into the assay vessel. This secondary antibody is antigen-specific also, but, in addition, bears a covalently-linked enzyme such as horseradish peroxidase. Since the quantity of enzyme present is related to the amount of antigen bound by the primary antibody, measurement of enzymatic activity permits an indirect quantification of antigen concentration.

The procedure is standardized by including known concentrations of antigen in each assay run. The *sine qua non* of this type of ELISA is that the antigen possesses a minimum of two antibody combining sites.

B. *Application of ELISA to Semen Stain Analysis*

At the present time, the reagents necessary to perform ELISA tests for p30 in suspected stains are not commercially available. Considering the levels of p30 in semen and its relative stability in dried semen stains, most forensic examinations would not warrant the detection sensitivity conferred by an ELISA technique. For these reasons, a detailed methodology will not be described here.

CALIBRATION OF p30 DETECTION METHODS

Quantification of p30 in semen stain extracts by RID or RIEP may be accomplished directly if one possesses gravimetric standards of this antigen. Unfortunately, such standards are not commercially available. Assuming that most forensic laboratory personnel have neither the time or expertise for purifying p30 from seminal fluid, the quantitative detection methods must be calibrated indirectly using a semen standard.

Indirect assay standardization can be accomplished by analyzing serial-doubling dilutions of a semen standard (commercially available) in parallel with the extracts of questioned specimens. The assay values (rocket height or ring precipitin area) given by the known semen standard dilutions are related to the reciprocal of the dilution. Assay values given by the unknown sample are then described in terms of the equivalent dilution of semen standard.

COMPARISON OF ASSAY PARAMETERS

Table 3 lists several parameters pertinent to each of the assay methodologies which might influence the choice of techniques. Those laboratories desiring only a qualitative test for the presence of p30 would select double diffusion or CIEP. If quantification of p30 levels is necessary for interpretative purposes, RID or RIEP techniques would be utilized.

Table 3. COMPARISON OF IMMUNOLOGIC TESTS FOR p30 IDENTIFICATION

Detection Method	*Sensitivity*[a]	*Time*[b]	*Cost/Assay*[c]
Double Immunodiffusion	10	24	0.15
Counter Immunoelectrophoresis	5	6	0.15
Rocket Immunoelectrophoresis	5	6	0.33
Radial Immunodiffusion	5	48	0.20
Enzyme-linked Immunoassay	0.001	12	4.00

[a]In μg p30/ml. [b]In hours. [c]In dollars

The sensitivities of all methods, except ELISA, are of the same order of magnitude. For CIEP, RID and RIEP, the lower limit of sensitivity may be reduced slightly by using a lower concentration of anti-p30 antiserum. One must remember that less antibody results in diminished amounts of immunoprecipitate and at some point the precipitate will become too faint to be visualized.

CONCLUSION

The intent of this monograph has been to educate the caseworking forensic serologist in several areas

germane to p30 detection in semen stains. A careful study of the analytical techniques available for p30 measurement should enable a cogent choice of assay method. A firm understanding of the biological and chemical characteristics of p30 as well as the principles underlying each of the assay techniques should suitably equip the analyst for rigorous cross-examination. Finally, it is hoped that laboratories which currently do not avail themselves of this powerful supplement to their semen stain identification protocol may be inspired to do so.

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DISCUSSION

Question: How stable is the antibody to p30?

Baechtel: I have never studied it, but antibodies are notoriously very stable, especially if they are kept in concentrated solutions. If you keep the protein content of the antibody solution up, you shouldn't have any problem with stability of the antibody.

Question: The number of times we have to use p30 to identify a semen stain on which we can't find sperm are relatively few and far between; so we keep p30 in the freezer. Does the continued freezing and thawing, at 2 to 4 week intervals affect the antibody's ability to form precipitate with p30 antigen?

Baechtel: Not in my experience, but I would recommend that your semen standards be divided into some aliquots which would be suitable. Then you could pull one aliquot out of the freezer and use it on a given test day, without having to thaw all the others.

Question: The freezing and thawing, of anti-p30 is a little violent; does that affect the antibody activity?

Baechtel: Once again, in my experience, no. If you dilute out your antiserum, and I am not sure why you would with the anti-p30, then you might get into some problems with instability upon repeated freezing and thawing. It is good laboratory practice to take your stock samples and aliquot them out into proportions that you think you would use for a given assay, so you can avoid repetitive freezing and thawing.

Divall: Have you tested postcoital vaginal material?

Baechtel: No, I have not. I do, however, think it is very important.

Divall: We seem to be avoiding a very important issue. Can I ask if anyone in the audience has tested p30 against postcoital, semen-free vaginal material?

Blake: I take it your concern is with the stimulated vagina. We haven't done quite the experiment that I think would be satisfactory, but we have collected vaginal material on tampons. If you would accept the proposition that a tampon in a vagina for a long period of time would provide, not quite the same degree of mental excitement, but perhaps some physical stimulation of the vagina, we have looked at a lot of those samples. Those samples have not shown any reaction with the antisera that has been prepared.

Divall: I think the response of the audience today is in agreement with the fact that we have developed several assays which we seem pretty definite about, as far as their ability to detect semen. However, I am beginning to wonder whether we have done sufficient testing on the material that is relevant. We must remember that every single time we develop a new antisera, that antisera has to be tested against all relevant materials. We can't elude back to the testing that was done on a batch of anti-p30 prepared last year. If we have a new batch of anti-p30, we have to test it against what is relevant.

Sensabaugh: In addition to the samples that Ed Blake referred to, we have done a couple with condom, postcoital collections using ELISA, and saw nothing. I feel reasonably good about that. Actually a more fundamental way of approaching this problem would be to determine whether or not the protein is present in any vaginal tissue. And there we would have to take a vagina, grind it up, and see whether we could detect anything. If it is not in any tissue, then we would not expect to see it in any fluid. I think if we could do that, then we wouldn't have to test every antiserum as suggested.

Baechtel: Of course problems of repetitively having to test different lots of antiserum could be obviated if we had a monoclonal antibody to p30. Then the specificity and titer would never change.

Sensabaugh: Are you aware of any work being done to develop a monoclonal antibody to p30?

Baechtel: The individuals at Roswell Park Memorial Institute in Buffalo, New York, have a monoclonal to p30 (prostate antigen). It is incredibly expensive.

THE IDENTIFICATION AND DISTRIBUTION OF ABH AND LEWIS SUBSTANCES IN SEMINAL AND VAGINAL SECRETIONS

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INTRODUCTION

Biochemistry of the ABO and Lewis Blood Group Systems

It was sometime after the discovery of the ABO group system before it was realized that the antigens were not confined to the red blood cells but were widely distributed throughout the body. Yamakami (1926) first demonstrated the presence of ABH substances in seminal fluid and saliva. Lehrs (1930) and Putkonen (1930) independently recognized that the character was dimorphic and that individuals could be divided into secretors and non-secretors.

Not all individuals who carry the ABH genes have the water-soluble blood-group substances in their secretions; the capacity to secrete ABH substances depends upon the inheritance of a gene at a different locus, the secretor gene. Classically those individuals who are hetero- or homozygous for the gene Se are secretors; those homozygous for the allele se are non-secretors as observed by Schiff and Sasaki (1932).

Watkins (1966) proposed that the function of the secretor gene is to regulate the expression of the H gene for, as we shall see later, H substance is a precursor of A and B substances.

The ABO System

It has long been recognized that the carbohydrate structures bearing A, B and H blood group specificities are part of glycoprotein molecules in secretions (Morgan 1960). The purified blood-group-specific glycoproteins are composed of 80 to 90 percent carbohydrate and 10 to 20 percent amino acids and contain many carbohydrate chains attached to a polypeptide backbone. The carbohydrate moiety consists of five sugars (Watkins 1978).

L-fucose
D-galactose
N-acetylglucosamine
N-acetylgalactosamine
Sialic acid

Before the isolation and characterization of the determinant structures, both Watkins and Morgan (1955) and Kabat and Leskowitz (1955) have indicated the importance in the secreted glycoproteins of:

N-acetylgalactosamine	— A activity
D-galactose	— B activity
L-fucose	— H activity

Current theories on the biosynthesis of blood-group substances (Watkins 1978) indicate that proteins are the direct product of gene action and it is the product of this gene action, enzymes, which are responsible for carbohydrate synthesis. These enzymes are known as glycosyl transferases and are involved in the conversion of a precursor substance to the specific products which appear in the blood-group substances.

The precursor substances comprise two different carbohydrate chains: Type I chain, where the terminal galactosyl unit is linked in the 1 to 3 position

Type I precursor chain

Gal *β1,3* GNAC *β1,3* Gal *β1,3* GalNAC

and Type II chain, where the terminal galactosyl unit is linked in the 1 to 4 position.

Type II precursor chain

Gal *β1,4* GNAC *β1,3* Gal *β1,3* GalNAC

Symbols: Gal — galactose
GNAC — N-acetyl glucosamine
GalNAC — N-acetyl galactosamine

The H gene controls the addition of L-fucose at the terminal position of both the Type I and Type II chains and, in secretions this conversion depends on the presence of the secretor gene (Watkins 1966).

H active structure

$$\begin{array}{l} \qquad \beta 1,3 \\ \text{Gal } \textit{or } \text{GNAC } \beta 1,3 \text{ Gal } \beta 1,3 \text{ GalNAC} \\ \qquad \beta 1,4 \\ \alpha 1,2 \\ \text{Fuc} \end{array}$$

The A gene controls addition of N-acetylgalactosamine in an alpha linkage to the terminal galactosyl unit of this structure to give A activity and, similarly, galactose is added to the terminal position of the H structure to give B substance.

A active structure

$$\begin{array}{l} \qquad\qquad\quad \beta 1,3 \\ \qquad\qquad\quad \text{or} \\ \text{GalNAC } \alpha 1,3 \text{ Gal } \beta 1,4 \text{ GNAC } \beta 1,3 \text{ Gal } \beta 1,3 \text{ GalNAC} \\ \quad \alpha 1,2 \\ \quad \text{Fuc} \end{array}$$

B active structure

$$\begin{array}{l} \qquad\qquad\quad \beta 1,3 \\ \qquad\qquad\quad \text{or} \\ \text{Gal } \alpha 1,3 \text{ Gal } \beta 1,4 \text{ GNAC } \beta 1,3 \text{ Gal } \beta 1,3 \text{ GalNAC} \\ \quad \alpha 1,2 \\ \quad \text{Fuc} \end{array}$$

Symbols: Gal — galactose
GNAC — N-acetylglucosamine
GalNAC — N-acetylgalactosamine
Fuc — fucose

The H active trisaccharides are identical to the A and B structures except that they lack the terminal N-acetylgalactosamine or D-galactose residue.

In the presence of the A and B genes, A and B activity is expressed on the red cells; but it requires the presence of the secretor gene before these blood-group substances are secreted in the body fluids. In the absence of the secretor gene, no blood-group substance is secreted.

The Lewis System

The Lewis blood-group system comprises two antigens, Le(a) and Le(b), which were first discovered by Mourant (1946) on the erythrocytes of a Mrs. Lewis. They differ from the ABH antigens in that they are not an integral part of the red cell membrane, but are absorbed from the plasma.

Grubb (1948) was the first to postulate that there was a link between secretor status and the Lewis groups found on the red cell. It was a year later that Grubb and Morgan (1949) showed that those people whose red cells typed as Le(a+) were non-secretors and those who typed as Le(b+) were usually secretors of ABH blood-group substances. Using inhibition studies of anti-Le(a) and Le(b), they also demonstrated the existence of Le(a) and Le(b) in saliva and suggested that the Lewis and secretor genes were part of the same system. It was not until almost 10 years later that Andersen (1958) postulated that there was an interaction between the ABH, Lewis and secretor genes. Andersen (1961) later put forward the theory that the Lewis and ABH receptors were formed from a single precursor substance.

We have previously seen that A and B substances cannot be secreted in body fluids without the prior conversion of a precursor substance to H; this conversion depends primarily on the presence of the secretor gene and secondly on the H gene.

However, the activity of the Lewis gene is not dependent upon either the H or Se genes and Le(a) substance is produced in secretions whether or not the H or Se genes are present.

Again we start with our precursor substance, but this time only with the Type I chain and in presence of the Lewis gene we get conversion to Le(a) substance. This involves the addition of L-fucose by an alpha 1 to 4 linkage to the sub-terminal N-acetylglucosamine residue of the Type I precursor chain.

Lewis a active structure

$$\begin{array}{l} \text{Gal } \beta 1,3 \text{ GNAC } \beta 1,3 \text{ Gal } \beta 1,3 \text{ GalNAC} \\ \quad \alpha 1,4 \\ \quad \text{Fuc} \end{array}$$

In the absence of the secretor gene no further conversion of the Le(a) substance occurs and only Le(a) will be detectable in the body fluids.

However, in the presence of the secretor gene, formation of the Le(b) activity structure takes place. Studies *in vitro* (Shen *et al.* 1968) have indicated that the Le(b) substance is formed in a two-stage process. The first stage involves the addition of L-fucose to the terminal galactose residue by the H transferase, and then the addition of a second fucosyl residue to the sub-terminal N-acetylglucosamine residue.

Lewis b active structure

Gal $\beta 1,3$ GNAC $\beta 1,3$ Gal $\beta 1,3$ GalNAC
$\alpha 1,2$ $\alpha 1,4$
Fuc Fuc

Symbols: Gal — galactose
GNAC — N-acetylglucosamine
GalNAC — N-acetylgalactosamine
Fuc — fucose

Thus, conversion of a Type I precursor chain by fucosyl transferase to give an H active structure can then either undergo conversion by the specific transferase to structures with A or B specificity or conversion to Le(b) substance.

When both the H and Le genes are active there is competition for the Type I precursor substance. When H substance is absent, for example in non-secretors, large amounts of Le(a) substance can be detected; but in secretors where H and Le(b) predominate, Le(a) substance will only be present in small amounts owing to competition for the substrate by the specific transferases.

To summarize the classical relationship between the ABH, secretor and Lewis systems:

	Lewis Group (red cells)	
SeSe	Le(a−b+)	ABH substances secreted
Sese	Le(a−b+)	ABH substances secreted
sese	Le(a+b−)	ABH substances NOT secreted

Although as we have seen there is a relationship between the ABH and Lewis system at a biochemical level, the Lewis locus is independent of both the secretor and ABH loci. This is illustrated by the existence of individuals whose red cell type is Le(a −b −): some do and others do not secrete ABH substances in their body fluids.

Methods Used for the Detection of the ABH Antigens in Body Fluids

The detection of A, B and H substances in blood and body fluids has been well documented over a long period of time. The three main techniques used for the detection of ABH and Lewis antigens in body fluids have been:

Mixed agglutination
Absorption-inhibition
Absorption-elution

The mixed agglutination method was first applied to the grouping of dried bloodstains by Coombs and Dodd (1961); its application to the detection of A, B and H group-specific substances in stains from body fluids was reported in 1969, but is not a method commonly used in the United Kingdom Forensic Science Service.

The classical method used for the ABO grouping of bloodstains was the inhibition test first described by Holzer (1931). The use of this method to test for secretor blood-group substances was described by Harley (1943). In this test antisera were added to extracts of the dried stain and, after a period of absorption, the residual sera were tested with the appropriate indicator cells. A reduction in the titer of the absorbed serum was evidence of the presence of the corresponding antigen. Although the method has changed little in principle since it was first used, in practice it has changed considerably.

The absorption-elution method was first described by Kind (1960). In this technique extracts of the stain were dried down in the wells of glass cavity slides before the addition of the antiserum. After a period of incubation any excess antibody was washed away and the indicator cells added. Elution of the absorbed antibody was carried out at 56° C and agglutination of the indicator cells was taken as evidence of the corresponding antigen. Modifications to the technique have been developed by a number of workers since that time (Nickolls and Pereira 1962; Outteridge 1962; Yada 1962; Budvari 1963; Fiori *et al.* 1963; Howard and Martin 1969)

At present there are two absorption-elution methods currently used in the United Kingdom Forensic Science Service. These are the extractive method after Kind (1960) or the single thread non-extractive method of Howard and Martin (1983).

While the extractive methods are simple and reliable, their main shortcoming would seem to be the need for larger amounts of stain than those used with single thread non-extractive techniques. In order to overcome this limitation, Davie (1979) reported a method where varying lengths of stained thread were extracted and ABO grouping carried out using the wells of a microtiter plate. It was found that the A and B antigens were easily detectable in 1 mm, and the H antigen in 5 mm, of bloodstained thread. Although a parallel study has not yet been reported for the detection of the ABH antigens in semen, this method has the potential as an alternative to the currently available techniques.

Each of the above techniques relies on the assessment of agglutination of control red cells as an indication that the antigen of interest is present. This assessment

is, by its very nature, subjective and the use of red cell suspensions can give rise to problems of reproducibility from day to day The development of an enzyme-linked immunosorbent assay (ELISA) for the detection of A, B and H blood-group substances is currently being undertaken at Central Research Establishment This technique has a colorimetrically measured endpoint to the reaction and thus is objective; initial reports show that it is more sensitive, in terms of both antiserum and the amount of material required, than the hemagglutination methods currently in use.

Methods Used for the Detection of Lewis Antigens in Body Fluids

The significance of investigating Lewis activity in saliva, semen and vaginal secretions was first reported by Pereira and Martin (1977). The method used was absorption-inhibition and was carried out in test tubes. Although in respect to the differentiation of secretors and nonsecretors, the Lewis grouping of body fluid stains was a major advance in forensic serology, there were certain limitations to its practical application. It is essential to use good quality antiserum capable of giving unambiguous reactions with the appropriate red cells. Difficulties arise as a regular supply of good Lewis antiserum is not easily obtained. Also, when it is available, it is generally used undiluted and thus its routine use is expensive. Davie (1980) reported that the use of microtiter plates in place of test tubes for the Lewis grouping of body fluid stains gave rise to both an increase in sensitivity of the method and economy in the use of antiserum. Using this method with his particular batch of antiserum, the optimal dilution for both the Le(a) and Le(b) was 1:32. In some laboratories this is now the preferred method for the Lewis grouping of body fluid stains.

ABO Grouping of Seminal Fluid and Semen Stains

Classically all individuals who are either homo- or heterozygous for the H gene will secrete H substance in their body fluids and individuals who have inherited the A and/or B genes will also secrete the corresponding blood-group substances. However, is this always the case? McNeil and his colleagues (1957) carried out a survey on the secretion of blood-group substances in saliva and found that this was not always a 100-percent rule and that there was a significant number of individuals of blood groups A and B who secrete H, but not A or B, substance and sometimes vice-versa. They termed such individuals as "aberrant" secretors. The saliva of several of these aberrant secretors were tested for Le(a) activity. One aberrant secretor who failed to secrete A substance was found to be Le(a) negative; whereas, all indications are that it should have been Le(a) positive. They further found that persons of blood group A_2 were unusually frequent among the aberrant secretors found in that study. Clarke *et al.* (1960) defined aberrant secretors as individuals whose A:H or B:H ratios fall more than a given number of standard deviations from the mean of a control group. They felt that such a definition would allow people in different places, using different antisera to compare their results. Smerling (1972) highlighted the problem that these "aberrant secretors" may present to the forensic serologist in the examination of seminal stains. For example, she reported a group B semen with H only detected by her inhibition method although both B and H were detected by mixed agglutination.

Interpersonal Variation in Levels of Secreted ABH Antigens

Dorrill *et al.* (1979) reported the results of a survey on the levels of ABH substances secreted in samples of liquid semen in an attempt to answer the following questions:

(1) What range of variation in ABH levels can be expected in a population?
(2) How does the proportion of A:H and B:H vary within that population?

A 3-channel Auto-Analyzer based on the hemagglutination inhibition assay by Sturgeon and McQuiston (1966) was used to assay A, B and H substances simultaneously. In the absence of an absolute standard, a relative standard was prepared from a large pool of secretor saliva and was allotted units of 256 units/ml of A and B and 512 units/ml of H. All the results were expressed in units/ml relative to this standard.

Two-hundred and twenty-five single semen samples from staff, hospital clinics and a local university were analyzed and it was found that the samples could be split into two discrete populations: those with detectable blood-group substances and those with none (nonsecretors). Seventeen percent of the semen samples tested had no detectable blood-group substances present. The results are summarized in Table 1.

The mean H level detected, regardless of ABO group, was very similar at around 22000 units/ml, but there were differences in the ranges of H substance detected from each group of individuals. Of the 109 Group O individuals, 10 had levels of less than 1000 units/ml, the remainder all falling between 9000 and 82,000 units/ml. Similarly in the Group A individuals, 3 of the 93 samples had very low levels of detectable H substance, i.e., less than 1000 units/ml. The remaining 90 samples fell within the range 5000 to 48,000

Table 1. MEAN AND RANGE LEVELS OF ABH BLOOD-GROUP SUBSTANCES IN SEMEN

Blood-group substances	*Units/ml*	
	Mean	*Range*
A	3940	20 -28500
H	21100	30 -48750
B	3140	150 -13500
H	22450	9500 -52000
H	23100	70 -82000

units/ml. The range of H substance detected from the Group B individuals was much smaller at 9500 to 52,000 units/ml, but these values are based on results from only 16 individuals. Had a larger number of subjects been studied, the range of levels observed would undoubtedly increase

The mean levels for A and B substances are much lower than for H at 3940 and 3140 units/ml, respectively. As a general rule there was little correlation between the levels of A and/or B and H substance. High levels of H may be associated with high or low levels of A or B substance. This feature is further emphasized when the A:H and B:H ratios are considered and the results are summarized in Table 2. From these results it can be seen that there is a large range in the A:H and B:H ratios and the mean ratio in both cases is much smaller than 1.0, i.e., more H than A or B substance detected. Sixty-one of the semen samples had an A:H ratio less than the mean of 0.264 and all but two of the remainder had ratios below 0.9. Only two of the 93 samples had more A than H substance detected and hence an A:H ratio greater than 1.0.

Table 2. THE PROPORTION OF A/H AND B/H IN SEMEN

	Mean	*Range*
A:H	0.264	0.003 -2.737
B:H	0.137	0.007 -0.289

The data for the B:H ratio was derived from only 16 samples, therefore neither the smaller range nor the lower mean value in comparison with the A:H ratio may be significant.

In this study 5 percent of the samples had low levels of A or B substance associated with high levels of H, and subsequently gave rise to low A:H or B:H ratios. It is such samples that are of particular interest to the forensic serologist as they may well be classed as aberrant secretors using the absorption-inhibition and elution techniques. The most extreme example of this was a sample of 150 units/ml A and 47,000 units/ml H. A sample like this could well give convincing results for A and H by absorption-elution, but H alone by inhibition. The knowledge that such individuals exist may help with the interpretation of such cases and emphasizes the advantages of performing absorption-inhibition and elution in parallel. For example, a sexual assault has taken place. The victim types as Group O, red cell Le(a -b +) and when the suspect is apprehended he admits the offense. His blood types as Group A, Le(a -b +). The seminal stains on the girl's clothing give a reaction for H by inhibition, but both A and H by elution. Although by using both methods this individual cannot be excluded as the possible source of the body fluid, using absorption-inhibition alone he could have incorrectly been excluded, even though he admitted the offense.

In this study it was shown that:

(1) There is a wide range in the levels of blood-group substances secreted in the semen of individual males.
(2) There is a wide range in the A:H and B:H ratios from different men.
(3) High levels of H substance may be associated with low levels of A or B substance.
(4) The A:H and B:H ratios were on average less than 1.0.

Intrapersonal Variation in Levels of Secreted ABH Antigens

Whitehead *et al.* (1981) reported the results of a quantitative study carried out on the levels of A, B, and H blood-group substances secreted in samples of semen collected from the same individuals over a period of 18 months. Samples were collected from 20 donors: 5 Group A, 2 Group B, 10 Group O secretors; and 3 non-secretors: 2 Group O, 1 Group A, as determined by Lewis grouping carried out on liquid blood samples collected from each. The number of samples collected from each donor varied from 3 to 20. In this study an attempt was made to answer the following questions.

(1) Are the levels of A, B and H blood group substances secreted in the semen of one donor constant with time?
(2) Are the A:H or B:H ratio for any one individual constant with time?

In this study the earlier findings that there was a wide range in both levels of blood group substances secreted and the A:H and B:H ratios found in a large number of different men were confirmed.

The levels of blood-group substances secreted by an individual over a period of 18 months were shown to be relatively constant in some but not all individuals. Examples are given in Table 3.

Table 3. LEVELS OF ABH BLOOD-GROUP SUBSTANCES SECRETED IN SEMEN OF INDIVIDUAL MALES OVER A PERIOD OF 18 MONTHS

Blood-group substance	*n*	*Units/ml*	
		Mean	*Range*
A	9	3664	2250 - 4600
H		6455	5000 - 7500
A H		0.565	0 450 - 0 729
A	20	434	148 - 2500
H		15776	10000 - 32000
A H		0 025	0 008 - 0.078
B	17	645	125 - 1450
H		11507	5850 - 15250
B:H		0.056	0.011 - 0 119

In the first donor, Group A, nine samples of semen were collected and it can be seen that the levels of A and H are relatively constant. The mean level of A substance is 3663 units/ml ranging from 2250 to 4600 units/ml. The corresponding mean level of H was 6455 units/ml with a range from 5000 to 7500 units/ml. In this individual the mean level of A substance detected was very close to the level of 3940 units/ml established from our population mean. However, the mean level of H substance detected was much lower than the population mean of 21,000 units/ml. Nevertheless, it did fall within the range of levels previously observed (Dorrill *et al.* 1979).

In another donor, also a Group A secretor, who provided 20 samples of semen, the mean level of A substance secreted was much lower than in the previous example at 433 units/ml ranging from 148 to 2500 units/ml. Once again the H levels were considerably higher with a mean level of 15,700 units/ml and a range of 10,000 to 32,000 units/ml.

In a third Group A donor, only three samples were collected and the levels of A substance detected were extremely low ranging from 185 to 513 units/ml. However, the corresponding levels of H substance were 6800 to 9400 units/ml.

In the 17 samples collected from the Group B donor there was considerable variation in the levels of both B and H blood-group substances secreted. The mean level of B substance secreted was fairly low at 654 units/ml ranging from 125 to 1450 units/ml. The corresponding mean level of H substance was 11507 units/ml with a range from 5850 to 15,250 units/ml.

Also in Table 3 we can see that in the two latter individuals there was considerable variation in the A:H and B:H ratios observed. In the former, the A:H ratios varied from 0.008 to 0.078 units/ml with a mean of 0.025, and in the latter, the mean B:H ratio was 0.056 ranging from 0.011 to 0.119 units/ml. In both these individuals neither the lowest level of A nor B substance detected was associated with the lowest level of H substance, confirming that low levels of A or B substance can be associated with high or low levels of H.

In view of the wide range of levels of A or B substance secreted by an individual over a period of time and the fact that sometimes the level is very low, it is important for the reasons previously stated to carry out both absorption-inhibition and elution in parallel. This possible variation in the levels of blood-group substances secreted by an individual over a period of time should be borne in mind when it is suspected that one individual is implicated in a number of sexual assaults carried out over a period of time. As both the United Kingdom and United States are now served by a good network of motorways it is not unknown for one person to commit such a series of assaults in more than one laboratory area. From the figures shown it is quite possible that an individual might secrete sufficient blood-group substances on one day to give good results by both absorption-inhibition and elution, but on a subsequent occasion to secrete sufficient to be detected by absorption-elution alone.

The levels of A, B and H blood-group substances detected in these individuals were obtained from samples of liquid semen. For the operational forensic scientist it is important to assess the potential problems of grouping body fluid stains containing low levels of A, B and H blood-group substances. With this in mind Shaw *et al.* (1981) undertook the manual grouping of stains prepared from some of the semen samples previously assayed. The samples were selected to include several which, by virtue of the low levels of ABH blood-group substances they contained, might be expected to present problems when grouped by absorption-inhibition and elution. The results of this study showed that few secretors will secrete A or B blood-group substances at a level that is not detectable by both the absorption-inhibition and elution techniques. However, there was a reduced ability of either technique to detect H substance and this could indicate a high rate of failure in the grouping of the stains originating from Group O individuals. It must be stressed that these results are based on the results of laboratory-prepared stains and may not necessarily apply to casework material.

Distribution of ABH Antigens Across a Seminal Stain

The problems associated with the grouping of saliva, semen and other body fluids is well recognized. One feature that was reported by Pereira *et al.* (1969) was that A, B and H substances were not evenly distributed

throughout the stain but tend to be concentrated in the inner part of the stain. This finding was confirmed by Rutter (1981) in his study on the distribution of the constituents of semen in stains. These findings are of practical importance when dealing with large stains In view of the small amounts of material used in the absorption-inhibition and elution tests, it may be possible to miss the area having blood-group substance activity present and hence, incorrectly assume that the stain came from a non-secretor. The value of Lewis grouping of body fluid stains is highlighted by the study of Rutter (1981), as he found that the distribution of Lewis substances did not coincide exactly with the ABH substances across semen stains. However, as was emphasized by Pereira and Martin (1976), the majority of stains present few problems and, provided the hazards are recognized and appreciated and suitable precautionary measures taken, the risk of erroneous results are minimized. In view of such problems, in their opinion both absorption-inhibition and elution should be used in parallel, incorporating dilution techniques, and results only reported when complete correlation is obtained. In addition, Lewis grouping was felt to be useful, particularly for the confirmation of non-secretor results.

The Lewis Grouping of Semen

In view of the problems associated with the supply of suitable Lewis antiserum, it is perhaps not surprising that to date no major quantitative study of the levels in body fluid samples has been undertaken. Bearing in mind the controversy as to the presence or absence of Le(a) from secretor semen, such a study must be considered to be long overdue.

Grubb (1951) was unable to detect Le(a) activity in the semen of 5 donors whose red cells typed as Le(a +), while Lodge and Usher (1962) demonstrated Le(a) substance in the semen of an Le(a + b −) donor and L(b) in an Le(a + b −) donor. Pereira and Martin (1977) reported that with regard to semen secretions, they were in general agreement with previous findings that Le(a) substance is absent from the semen of Le(a − b +) men. However, they did find examples of secretor seminal stains in crime investigation which gave a positive reaction for Le(a), although the possibility of another source of Le(a) in these stains could not be categorically excluded. Piner and Sanger (1980) reported the presence of both Le(a) and Le(b) in semen stains from a Le(a − b +) individual. Their findings were confirmed by Hossaini and Fisher (1981). Hossaini also reported differences in the concentration of Lewis substances in different individuals. Where both Le(a) and (b) were present in a semen sample, Le(b) was present in considerably higher concentrations than Le(a). The titer of Le(b) in the individual samples varied by 2 doubling dilutions from 1:128 to 1:512. Corresponding titers for Le(a) were much lower at 1:4 to 1:16 when associated with Le(b), but were much higher in Le(b −) individuals at 1:128 to 1:256.

This initial report by Hossaini and Fisher (1981) on levels of Le(a) and Le(b) in semen was carried out on 11 samples using a manual absorption-inhibition method. The development of a more reliable source of antiserum and a quantitative method would be an important development in estimating the variation in Le(a) and Le(b) activity in the semen of a large population. We must hope that with the potential availability of monoclonal anti-Lewis serum we can come some way to achieving this aim.

ABH Grouping of Vaginal Swabs

In sexual assault cases one of the most important exhibits submitted for examination is the vaginal swab which is taken after the offense has been committed. Once the presence of semen has been confirmed on the swab, it is then usually necessary to detect any ABH antigens present in order to include or exclude a suspect.

The first report of ABH grouping of vaginal swabs was made by Masis (1967). He reported that there was sufficient ABH substances in vaginal secretions to be easily detected. Since this time, although vaginal swabs have been typed routinely for the presence of ABH substances, there is very little literature relating to the variation in levels of ABH and Lewis substances that can be expected:

(a) from different women,
(b) from the same woman over the course of a menstrual cycle,

and this is undoubtedly due to the difficulty in obtaining vaginal samples.

In 1983 Martin and Cheshire at the Metropolitan Police Forensic Science Laboratory published a report in which they investigated the levels of A, B and H blood-group substances secreted by two sexually inactive donors throughout the course of one menstrual cycle. Swabs were collected from one Group A and one Group B secretor. The amount of blood-group substances secreted by both donors was found to vary considerably throughout the menstrual cycle. In the Group A donor, almost no inhibition of the antiserum was obtained from swabs taken on days 9 and 12, while high levels of inhibition were observed from other swabs taken in the middle of the cycle. Similarly with

the Group B donor, high levels of inhibition were obtained with some swabs but at least 7 of the swabs showed low blood-group B activity.

Hossaini *et al* (1981) reported that in samples of vaginal secretions obtained from three Group A and two Group B females, the levels of A or B substance detected was higher than that of H. In addition, they showed that the levels of A, B or H substances secreted in vaginal fluid were greater than that of the corresponding saliva sample, a feature also reported for both Le(a) and Le(b) substances.

These two studies illustrate a feature that is well recognized in casework: that the levels of ABH substances secreted in vaginal fluid are sufficient, at times, to make the interpretation of grouping results from mixed seminal/vaginal stains or swabs difficult. Davies (1982) reported a number of examples; a victim was raped by a male "friend" while returning from a dance. The vaginal swab gave reactions for the victim (Group A secretor) but her pants for Group AB secretor. The "friend" was a Group B secretor. Such examples of unexpected results of ABO grouping on swabs emphasize that considerable caution must be exercised in the interpretation of grouping results of seminal stains contaminated with vaginal material.

Although valuable information can be obtained by grouping vaginal swabs when the victim and assailant are of different ABO groups, no conclusions as to the group of the semen can be drawn if:

(1) The suspect is a non-secretor.
(2) The suspect is a Group O secretor and the complainant a secretor of any group.
(3) Both individuals share that same ABO Group and secretor status.
(4) The complainant is an AB secretor.

The Lewis Grouping of Vaginal Swabs

The first report of Lewis grouping of vaginal secretions was made by Pereira and Martin (1977) who found that Le(a) substance was not detected in vaginal secretions from Le(a–b+) women. In contrast to this finding, Piner and Sanger (1980) reported that a vaginal swab from a secretor, i.e., Le(a–b+), can be expected to show both Le(a) and Le(b) activity, a finding confirmed by Hossaini *et al*. (1981). Further results by Hossaini *et al*. (1981) on the relative concentrations of Lewis substances in vaginal secretions showed that Le(b) was secreted in higher amounts than Le(a) and that the levels were variable. Where both Le(a) and Le(b) were present, Le(b) was present in higher concentrations to Le(a), but when Le(a) alone was detected then the levels were much higher.

Further results by Hossaini on a study o concentrations of ABH and Lewis in vagi showed that 18 of the 116 swabs studied g ing, unexpected results. These results i absence of A and H blood-group subst Le(a–b+) individual, the detection of stance from two different Le(a–b–) wo detection of small amounts of Le(b) in a subject. Despite these conflicting resul indicated like saliva, vaginal secretions fre often contain a high titer and concentrati more of the ABH and Lewis substances in with their red-cell blood group and Lewis p

Some of these discrepancies may be acc by the fact that it was assumed, but not asc questioning, that the subjects had not intercourse for at least 24 hours before t were taken.

Thus, from these results it is evident that assume that the presence of Le(a) substance seminal stain or a vaginal swab is indica involvement of a non-secreting individual.

The value of Lewis grouping of seminal vaginal swabs can be realized if:

(1) Non-secretor ABH results can be v finding a stain to be Le(a+b–).
(2) A victim is a non-secretor, i.e., Le(a– presence of semen from a secretor can by demonstrating Le(b) activity.
(3) The victim is Le(a–b–), the presence from either an Le(a+b–) or Le(a–b+ lant could be identified.

So to summarize:

(1) There is a large range in the levels of A, H blood-group substances secreted in t of different men.
(2) The levels of blood-group substances s the semen by a particular individual does not always, vary considerably.
(3) In semen very low levels of A and B blo substances can be associated with very els of H substance and such samples rise to grouping problems using manua tion-inhibition.
(4) In the Lewis grouping of semen and secretions both Le(a) and Le(b) substan been detected in secretions from indi whose red cells type as Le(a–b+).
(5) The presence of Le(a) substance cannot t be considered to be definitive for the ment of non-secreting individuals.

In view of the problems already outlined United Kingdom Forensic Science Servic

absorption-elution and [illegible]

in parallel incorporating a dilution technique, and when Lewis grouping is carried out both Lewis (a) and (b) are used and the results only reported when complete correlation between the results are obtained.

In practice the vast majority of stains appear to present few problems and, provided the hazards are appreciated and suitable precautionary measures taken, the risk of erroneous results should be minimized.

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DISCUSSION

Blake: How does one relate the titer units one gets from the auto analyzer to those obtained through the more classical manual methods?

Dorrill: The units allocated to our standard was based on our attributing 256 units per ml of A and B, and 512 units per ml of H. These units were based on the inhibition titers that were obtained for that saliva pool using a manual inhibition technique.

SURVEY REPORT: SEXUAL ASSAULT RESEARCH AND INVESTIGATIVE EFFORTS OCCURRING IN FORENSIC SCIENCE LABORATORIES

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For many years the forensic biochemists and serologists have devoted much of their research time to the investigation of genetic markers which are present in blood. Methods and techniques emerged which were capable of detecting protein and enzyme polymorphisms and antigenic differences in dried bloodstains.

During this time the necessity to develop methods which could be used by laboratories not equipped with sophisticated apparatus became apparent.

In recent years the emphasis has moved from bloodstain investigation and the identification of sexual fluids, to the detection of genetic markers in these fluids.

The scientific investigation of sexual assault cases has attracted considerable interest and has already been the subject of a British Academy of Forensic Science Symposium and a Haemogenetics Society meeting in the United Kingdom (UK) during 1982. It was obvious from these meetings that a large amount of international research is being devoted to this subject. Some of the findings have shed light on problems which have concerned us for years, other findings have given conflicting results and some have given us cause to rethink our original understanding.

In this brief review I shall attempt to discuss the investigations which have taken place, the methods which are in current use and research work which is in progress at the present time. I have deliberately omitted a large amount of the research taking place in the United States because that will be covered by other contributors to this symposium.

The research and development which is carried out falls broadly into two categories:

1. The identification and characterization of sexual fluids, especially when they occur as mixtures and also when they are in the form of dried stains.
2. The detection and typing of genetic markers which are present in the sexual fluids.

Identification of Sexual Fluids

Acid phosphatase has been used for many years as a preliminary diagnostic feature for the presence of semen. Vaginal secretion also contains an acid phosphatase and, if a quantitative enzymic assay is used, it is necessary to establish a level of confidence above which it can be assumed that the acid phosphatase originated from semen. However, this makes the assumption that acid phosphatase levels from postcoital vaginal secretions can be adequately measured.

It has become apparent that the seminal and vaginal acid phosphatases are the same or very similar enzymes and forensic scientists are faced with a dilemma when one group of workers claims to have an antibody which is specific for prostatic acid phosphatase (Vihko *et al.* 1981), while another author shows that antibodies raised against pure prostatic acid phosphatase are not specific for the male enzyme (Dissing 1981).

Minor differences may occur between the vaginal and prostatic acid phosphatases and it depends on the ability of the rabbit's immune system to detect those differences and produce a specific antibody. It may transpire that monoclonal antibodies will provide the answer.

Baxter (1973) developed an immunorocket technique and this together with subsequent radioimmunoassay techniques require the use of an antibody specific to semen.

Adams and Wraxall (1974) described an electrophoretic method for the specific determination of prostatic acid phosphatase. Unfortunately, this method is not free from problems as many workers have found that the electrophoretic mobilities of prostatic and vaginal acid phosphatases will vary under certain conditions and one can be confused with the other.

At the Metropolitan Police Forensic Science Laboratory (MPFSL) we have shown that vaginal acid phosphatase levels can be elevated postcoitus (Figure 1) and this introduces yet another problem. Although we have only studied a few donors we must assume at this stage that there could be a considerable increase in the postcoital levels of vaginal acid phosphatase from some women and thus an even more confusing situation will develop. Work is in progress to try to obtain a value for this increase.

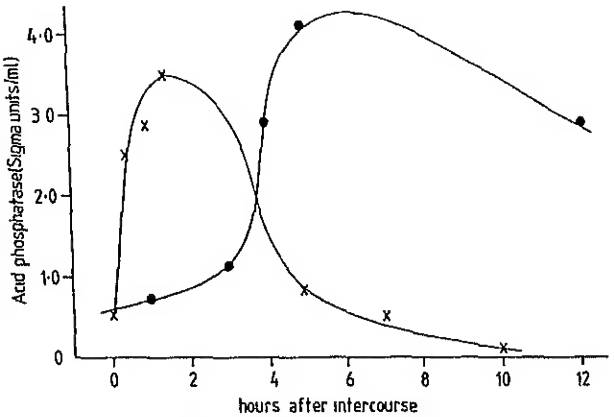

Figure 1 Acid phosphatase levels from extracts of postcoital semen-free, vaginal swabs.

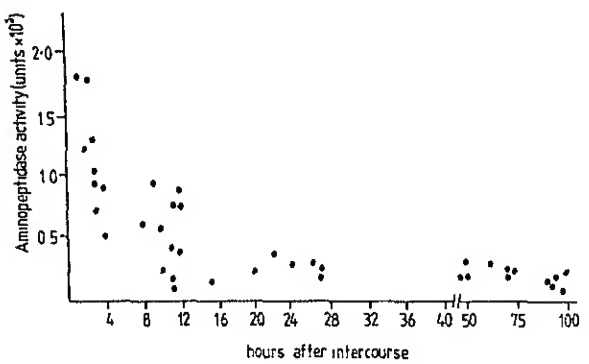

Figure 2 Aminopeptidase activity from vaginal swabs taken after intercourse using L-leucyl β-naphthylamide (1 unit = 1 μ mole of β-naphthylamine produced per minute, per ml of sample)

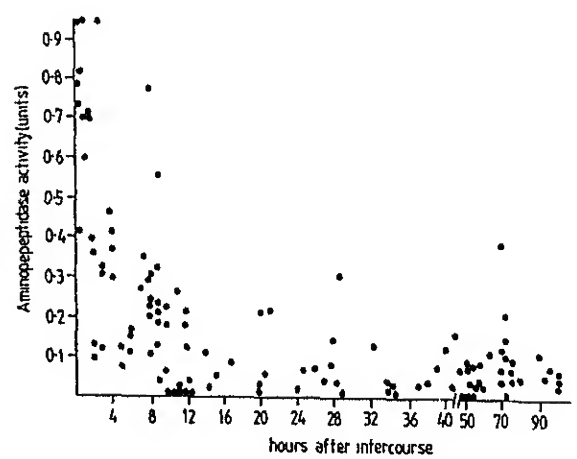

Figure 3. Aminopeptidase activity from vaginal swabs taken after intercourse using l-leucyl p-nitroamilide (1 unit = 1 μ mole p-nitroaniline produced per minute, per ml of sample)

Oya *et al.* (1978) suggested the use of a quantitative measurement of leucine aminopeptidase (LAP) for distinguishing between semen and vaginal secretion in the absence of spermatozoa. Lawton and Sutton (1982) in a follow-up study concluded that it was not desirable that testing for LAP should replace testing for acid phosphatase as an initial screening test for semen. At the MPFSL we found that the assay method suggested was not in fact specific for LAP but would detect a whole range of peptidases. The overall study which we carried out provided a rather similar pattern (Figure 2 and Figure 3) to that obtained from postcoital acid phosphatase concentrations when p-nitrophenyl phosphate was used as a substrate. We would also not advocate the use of aminopeptidase assays to replace those of acid phosphatase, but the two could be used together.

The real breakthrough in the search for methods of distinguishing sperm-free semen from vaginal secretion emerged with the description of p30 (Sensabaugh 1978) as a specific indicator of semen. Although this protein had been previously described by Li and Beling (1973), it had gone largely unnoticed in the forensic science community.

At the MPFSL we have now completed one study of a comparison of p30 with acid phosphatase levels (Figure 4 and Figure 5) and are now recommending its use in our laboratory (Poyntz and Martin 1984). However, we add a cautionary note in that during the study we were only able to test the antisera against a few semen-free, postcoital vaginal swab extracts and for the present we cannot dismiss the chance that a reaction with anti-p30 may yet be found with vaginal secretion.

This subject will be dealt with much more effectively in another part of this conference.

There may be times when the forensic biologist wishes to identify vaginal secretion, but as is normally the case, the question arises as to whether one has a mixture of semen and vaginal secretion such that any genetic markers found in the stain can be assigned to one or both of the donors. The specific identification of vaginal secretion has presented an even bigger problem than that of the semen.

As far as I am aware there is nothing which is solely specific to vaginal secretions, but the following tests could help to provide an indication as to the presence of this fluid.

Throughout the menstrual cycle the epithelial cells produce an excess of glycogen which is anaerobically metabolized by commensal lactobacilli. The resultant lactic acid formed, as a metabolite, lowers the pH of

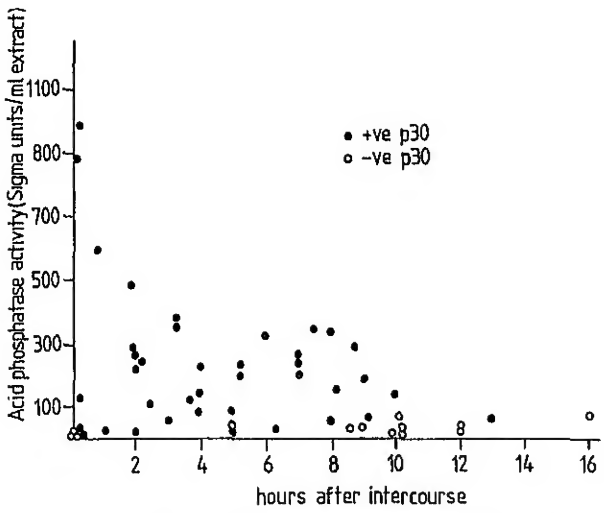

Figure 4 p30 detection from donor vaginal swabs taken at various times after intercourse.

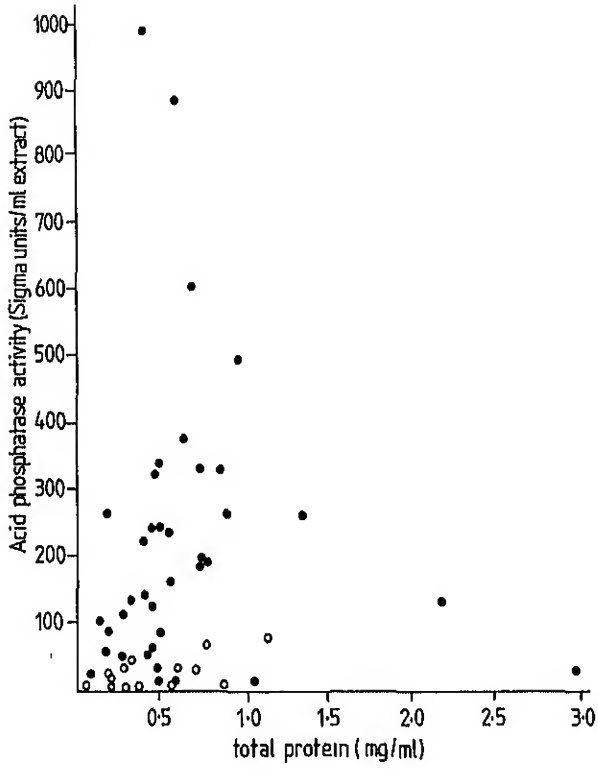

Figure 5. Detection of p30 from donor vaginal swab extracts with relation to total protein.

the vagina and inhibits the growth of other micro-organisms which may be pathogenic.

This high level of glycogen has been used as a feature of vaginal epithelial cells, whereby iodine is interchelated by the glycogen and the cell takes on a brown coloration.

Although this test has been used for many years and provides a very good indication of vaginal secretion, it is not necessarily specific and the lack of brown color does not mean that vaginal cells are absent.

The identification of vaginal microflora have been tentatively suggested as a means of demonstrating vaginal secretion but the difficulty with obtaining viable organisms for accurate identification has always been a major problem.

Divall, at the MPFSL, has identified a peptidase which is determined electrophoretically and is apparently vaginal in origin (Figure 6). At the moment the best that can be said, if this peptidase is found, is that there are very strong indications of the presence of vaginal secretion, as he has failed to detect this peptidase in all other body fluids tested. However, if the peptidase is not found, it cannot be assumed that vaginal secretion is absent.

During some studies in which we were attempting to identify proteins of semen and vaginal secretion we were struck by the levels of lactate in vaginal secretion and citrate in semen. Although this has been extensively reported in the literature, we could not find a study which has compared the relative levels of the two carboxylic acids in the sexual fluids. Therefore, we are carrying out a survey of extracts from seminal stains, vaginal swabs (semen-free) and vaginal swabs bearing semen in order to determine the levels of lactate and citrate present (Figure 7). Isotachophoresis is a particularly good technique for this study because the extracts require no preliminary treatment and the analysis of each sample takes only about 15 minutes. So far we have found the method relatively easy to operate and have successfully determined the carboxylic acids in the respective fluids, but as yet insufficient data is available to report it as a reliable method. A very good explanation of the theory and application of isotachophoresis was presented by Holloway and Trantschold (1982).

The female ejaculation following stimulation of the Graffenberg spot does not appear to have attracted scientific interest in the UK and Europe.

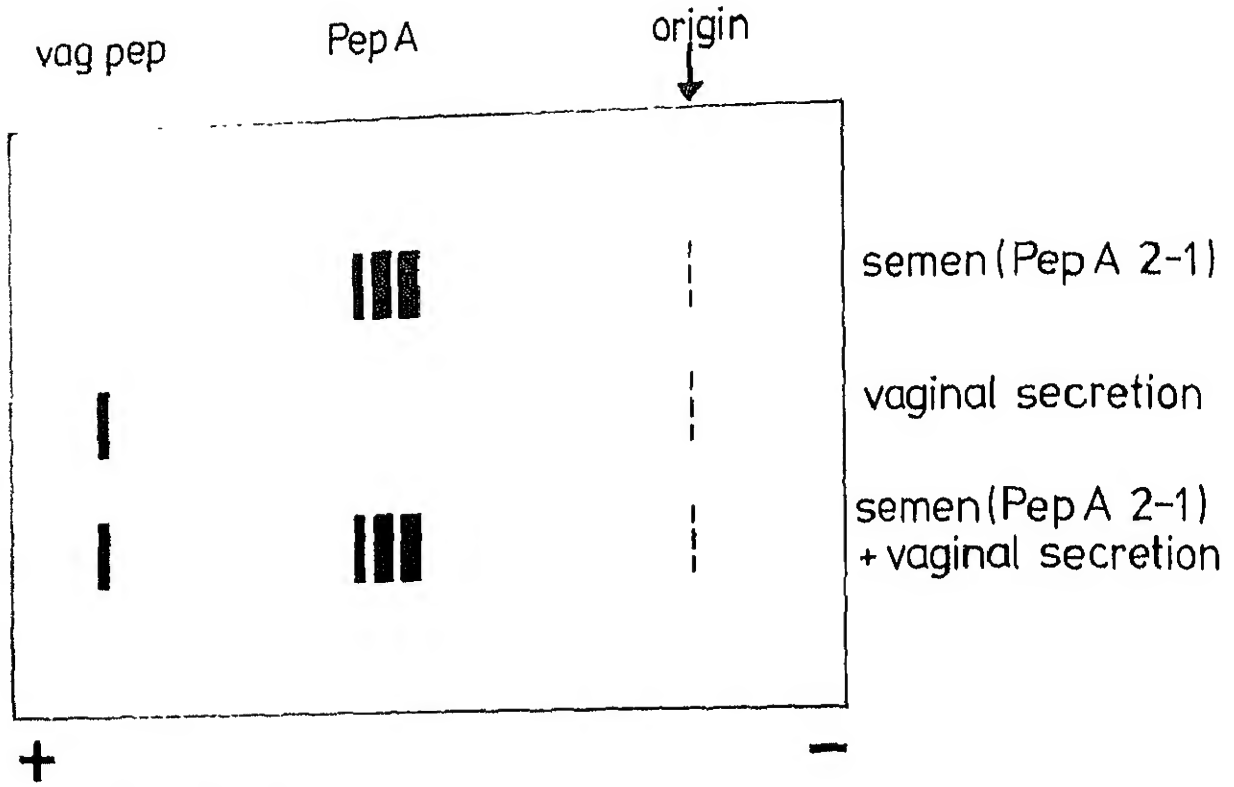

Figure 6 Diagrammatic representation of the electrophoretic separation of Peptidase A and vaginal peptidase.

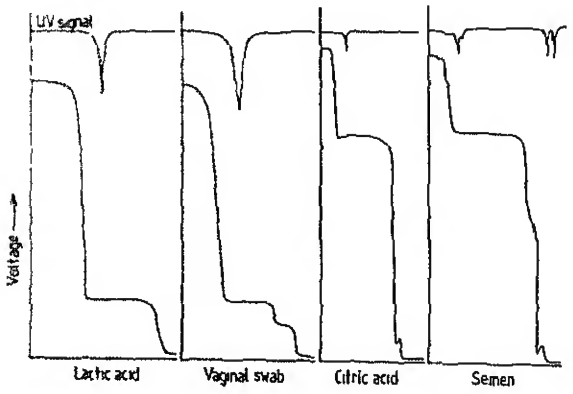

Figure 7 Detection of lactate and citrate ions from vaginal secretion and semen using isotachophoresis

Genetic Markers in Sexual Fluids

Many techniques have been reported for the typing of the ABH antigens in body fluids and, as with most other systems used in forensic science, they are not without conflict.

At the MPFSL we still use an inhibition technique and an elution technique in parallel and unless there is a correlation between the two results we report an inconclusive finding (Pereira and Martin 1976).

The necessity for this correlation became apparent to us recently when carrying out a study of vaginal swabs from donors in the laboratory. The work was a collaborative effort between Dr. Lincoln at the London Hospital and ourselves (Davies *et al.* 1984).

The male involved was typed as group A, Le(a−b+), and the female was typed as group O, Le(a+b−). The inhibition results obtained from the vaginal swab extract gave group A reactions but the elution results showed strong group AB reactions (Table 1). This phenomenon was observed from swabs which were taken up to three hours after intercourse. After that length of time the group B activity could not be demonstrated (Table 2). The initial results were obtained using the routine typing sera where the anti-B had an initially high titer and was diluted for use in the inhibition technique. Subsequently, a low titer anti-B was used which was successfully inhibited by the vaginal swab extract.

A semen sample was obtained from the male donor and this proved to be the source of the group B reactions.

On further testing, using a monoclonal anti-B serum, we were unable to demonstrate any group B activity by elution (Table 2).

Some microorganisms were isolated from the semen

Table 1. RESULTS OF ABH GROUPING OF A POSTCOITAL VAGINAL SWAB. THE LACK OF CORRELATION BETWEEN THE INHIBITION AND ELUTION RESULTS INITIATED SUBSEQUENT WORK TO DETERMINE THE ORIGIN OF THE ABERRANT B REACTION OBTAINED BY ELUTION

Dilution of Swab Extract	*anti-A*	*anti-B*	*anti-H*
INHIBITION			
neat	—	4	—
1/5	—	4	—
1/10	—	4	—
ELUTION			
neat	—	4	—
1/5	4	4	2
1/10	4	4	3
1/20	4	4	4
1/40	4	4	4

Table 2. RESULTS OBTAINED FROM ELUTION STUDIES OF THE EXTRACTS OF POSTCOITAL SWABS. THE ABERRANT B REACTIONS COULD NOT BE DETECTED AFTER 3 HOURS USING ROUTINE TYPING SERA AND NO REACTIONS WERE OBTAINED WITH THE MONOCLONAL ANTI-B

	anti-B	*monoclonal anti-B*
Pre-intercourse	—	—
Immediately postintercourse	2	+
10 minutes	3	—
1 hour	2	—
3 hours	+	—
38 hours	—	—

sample but no blood-group activity could be attributed to them.

During this investigation we encountered two casework samples which also appeared to be giving aberrant group B reactions.

Therefore, we are now particularly careful when interpreting the results from the ABH typing of body fluid stains.

When mixtures of these sexual fluids are encountered it has always been a matter of conjecture as to the level of ABH activity contributed by the vaginal material. We therefore instigated a study to determine the variation in the quantity of A or B blood-group substance present in the transudate extracted from vaginal swabs, which were donated by women who abstained from sexual intercourse for the duration of the experiment.

Originally two female donors (one group A secretor daily basis throughout one complete menstrual cycle. Typing was carried out using inhibition techniques both manually and with an autoanalyser.

The results showed considerable variation throughout both of the cycles and no obvious pattern emerged (Figure 8 and Figure 9). The work was criticized on the basis that the level of ABH activity could be due to intrinsic changes in secretion or to the amounts of secretion removed onto the swabs.

Therefore, we carried out a second set of tests in which ABH activity and total protein were assayed simultaneously. In this case we found that both the ABH activity and the protein levels were variable but

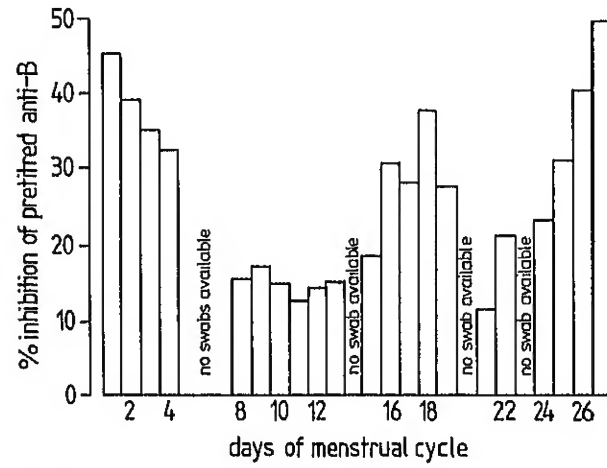

Figure 8. Group B activity from vaginal swabs taken from one donor throughout one menstrual cycle. Blood group activity was determined by an inhibition technique using an autoanalyzer.

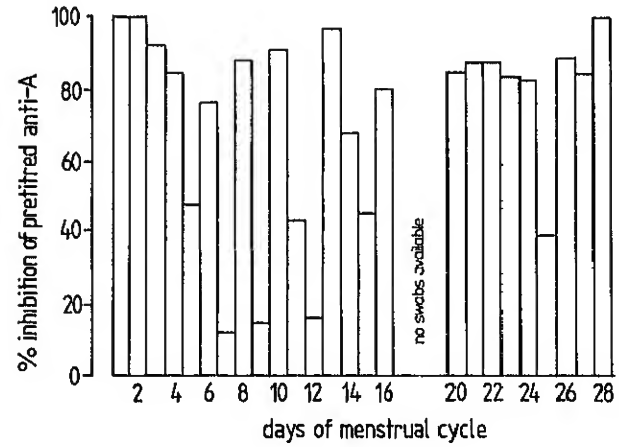

Figure 9. Group A activity from vaginal swabs obtained from one donor through one menstrual cycle. Blood-group activity was determined as in Figure 8.

that there was no correlation between the two (Figure 10).

Therefore, we concluded that if a scientist was presented with a vaginal swab from a group A secretor woman with semen present from a group A secretor man, it would be impossible to determine how much group A substance originated from the man and how much was contributed by the woman.

The way in which the swabs are taken is very important and we have also demonstrated that it is virtually impossible to obtain duplicate swabs (Figure 11).

The question of whether the secretion was high in group A substance or a lot of secretion was removed during the sampling becomes irrelevant because the scientist would have no way of knowing this in a casework situation, as he is not involved with the sampling procedure.

The Lewis grouping can often help in the interpretation of the ABH results obtained from vaginal swabs or stains of sexual fluids, but many forensic science laboratories have reported incompatible Lewis grouping results.

For our routine testing of stain extracts (at the MPFSL) we prepare a dilution series for our inhibition testing using pretitred Lewis antisera.

Recently we found that the particular batch of anti-Le(a) which we were using was not compatible with the anti-Le(b) in that one could be inhibited by soluble Lewis substance far more easily than the other. In this situation if the dilution series of the extract was not

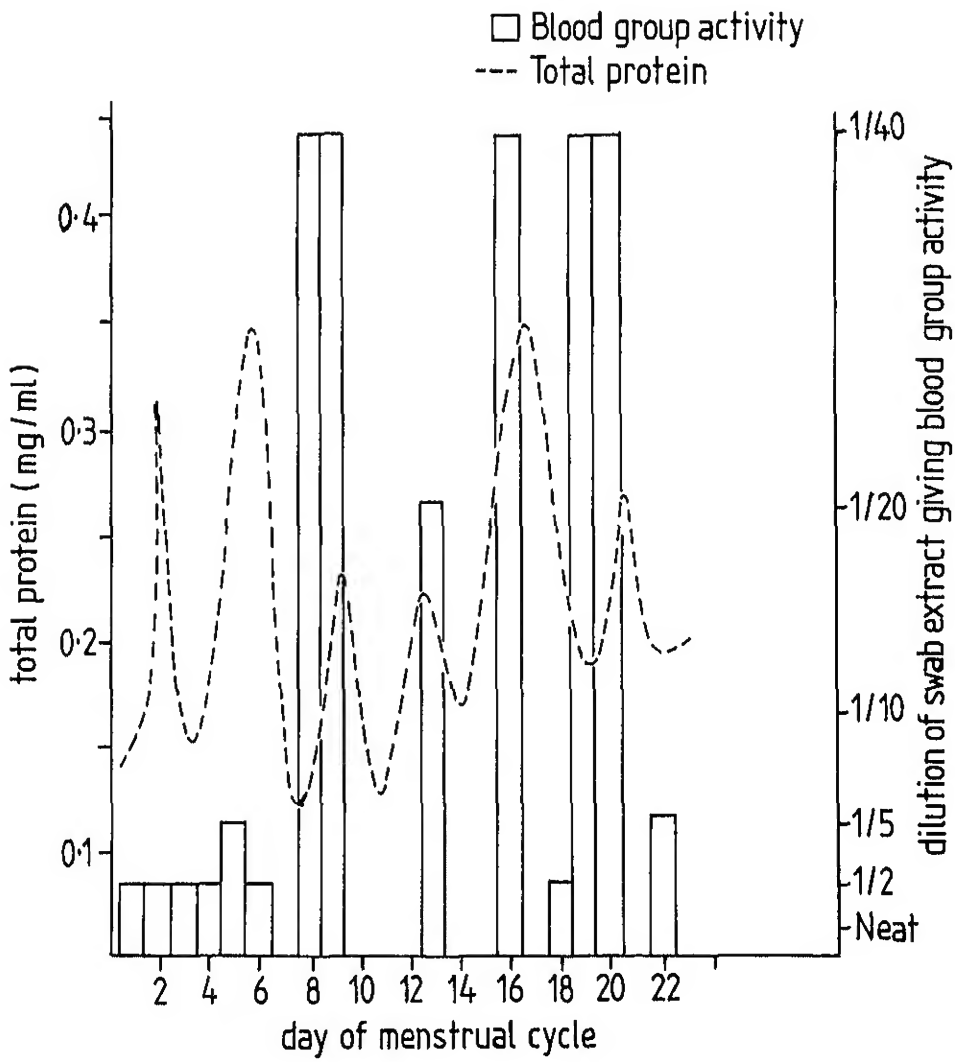

Figure 10. Group B activity and total protein from vaginal swab extracts taken from one donor throughout one menstrual cycle. Blood-group activity was measured using absorption-elution from dilutions of the swab extracts. Total protein was determined using a modified Lowry method.

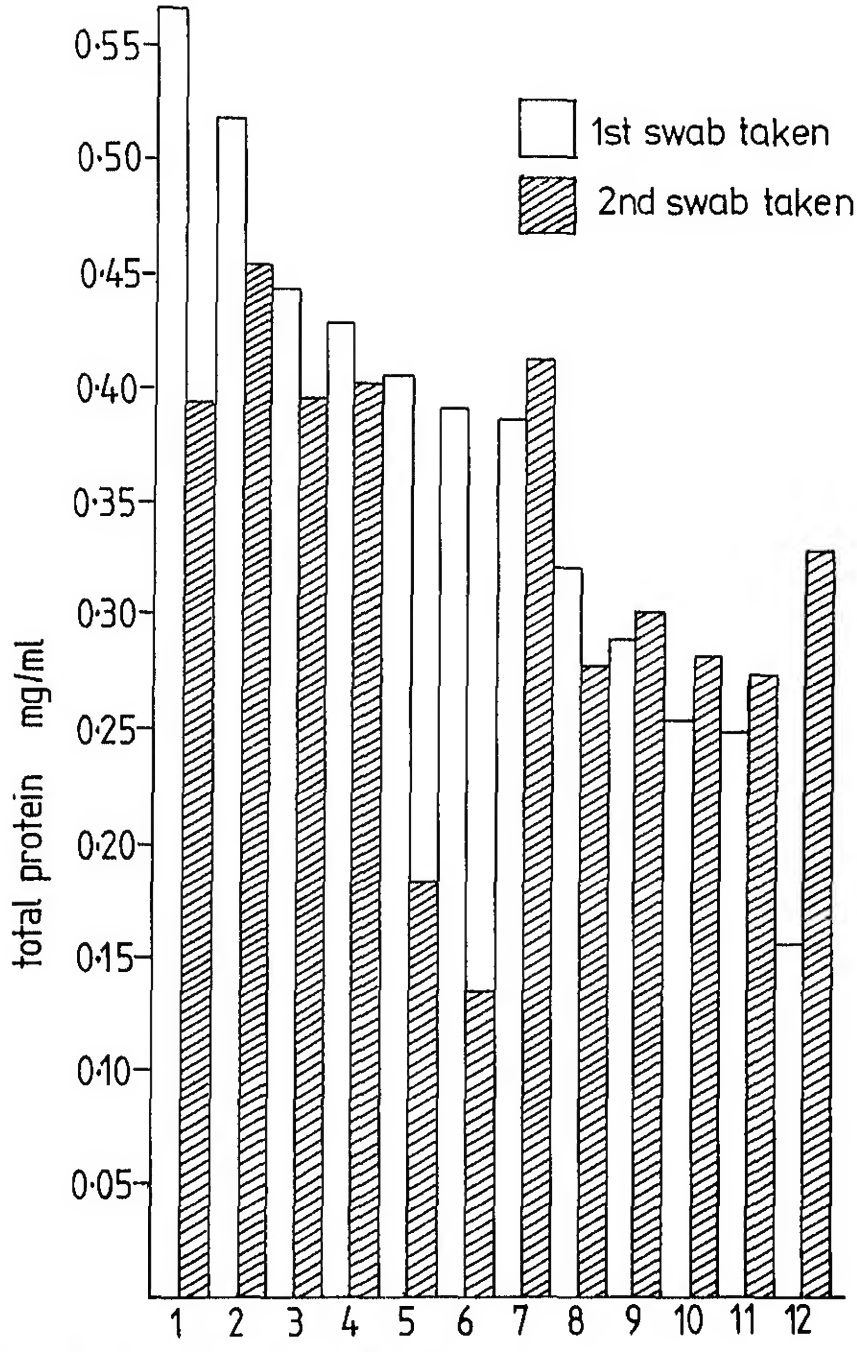

Figure 11. Paired vaginal swabs taken from different donors. Each swab was extracted into 1 ml of distilled water and the protein content measured by a modified Lowry method

adequate an error in typing could occur (Table 3). The situation has been resolved by more stringent testing of the antisera before use in the routine typing of casework material.

The initial euphoria over being able to detect the Gm antigens in semen has somewhat abated with the realization that it is a somewhat difficult system to use in casework. A relatively large amount of stained material is required to give a meaningful result and naturally occurring antibodies, which will agglutinate the indicator cells, are frequently found.

In a large number of cases the female Gm groups from the vaginal secretion are more easily detected than those from the semen.

TABLE 1 INHIBITION RESULTS OBTAINED FROM A DRIED SALIVA STAIN DONATED BY A PERSON WHOSE RED CELLS TYPED AS Le(a-b+) USING INCOMPATIBLE ANTISERA. IN THIS INSTANCE IF DILUTIONS 1/5 AND 1/10 WERE USED A MIS-TYPING WOULD OCCUR

[illegible]	anti-Le(a)	anti-Le(b)
[illegible]	--	--
1/2	--	--
1/5	--	3
1/10	1	4
1/20	4	4
1/40	4	4

The Km(1) antigen presents a particular problem because, in the absence of a positive inhibition result, it is impossible to say whether the antigen is truly absent or if there is insufficient material to determine its presence

As with the Lewis system the selection of antisera, which are of sufficient caliber to be used for Gm/Km determination from stained material, becomes very important Consequently, these antisera have to be fully characterized each time a new batch is purchased, and this can be a costly and time-consuming business.

Hodge *et al.* (1981) described a method for the typing of some HLA antigens in bloodstains and, with the reports that these markers were also present in semen, it appeared to us that this could be a very useful system to adapt to the grouping of the sexual fluids.

We experienced many problems with typing bloodstains (Martin 1981) mainly due to the commercial reagents which we had to use. In addition, because of the report at the Haemogenetics meeting (English Speaking Group 1980) that a group of Dutch workers were unable to confirm the presence of the HLA antigens in semen, we abandoned the project.

An interesting new development occurred with the presentation by Panari and Guisti (1971) of a secondary polymorphism in the ABO system which could be detected in saliva and semen. The method required the use of Sephadex G-100 gel filtration from which three fractions could be obtained. In separate samples of semen varying combinations or absences of the three fractions could be found, and the seminal gel filtration pattern obtained was thought to be stable and genetically controlled. Unfortunately, a group of workers at the Central Research Establishment in the UK were unable to reproduce this work and concluded that the polymorphism did not, in reality, exist.

PGM grouping of vaginal swabs and seminal stains has been used for some considerable time and it has been noted that the female group can be determined more easily after intercourse than before.

Parkin (1977) demonstrated that the glyoxalase phenotype of a male donor could only be found on vaginal swabs which were taken within two hours of sexual intercourse. After this time an uninterpretable glyoxylase pattern was often seen, and between two to ten hours postcoitus the vaginal glyoxalase phenotype was generally observed with no trace of the semen phenotype. He hypothesized that proteolytic enzymes in the vagina would break down the semen glyoxalase after about two hours. The product of this partially degraded glyoxalase plus the vaginal glyoxalase would produce the uninterpretable pattern.

The vaginal glyoxalase could be produced as part of the mechanism of tissue regeneration following intercourse.

He concluded that only swabs taken within two hours of sexual intercourse could be grouped. These findings have led us to believe that the biochemistry of the postcoital vagina is somewhat different from that of the inactive condition. We have found that the levels of AP and PGM are elevated after intercourse, but we are hampered by the lack of donors to provide swabs for this work. The reason for this becomes obvious when one reads the instructions for obtaining the swabs. A swab must be taken before intercourse, the male partner must use a sheath type contraceptive during intercourse and swabs must be taken very soon after intercourse and at subsequent timed intervals preferably every hour up to 12 to 14 hours.

However, with all of the casework and research problems, we feel that it is absolutely necessary that all of the investigations are carried out with some tests being made on semen-free, postcoital vaginal material.

With our present knowledge it is not possible to predict with certainty what changes will be found in the biochemistry of the postcoital vagina, but there are various indications that there is an increase in the level of enzyme activity and it may be that the antigen levels are also elevated. We can offer no easy solution as to how to obtain the necessary samples.

At the moment there are a number of research workers who are actively involved with the study of the forensic investigation of sexual assault, but the subject has still not received any clear indication of a direction in which we should go to obtain unequivocal answers.

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DISCUSSION

Question: I have a general question about the direction of research. It has to do with a half-life of materials in seminal/vaginal mixtures and the fact that they had been studied now extensively *in vitro*. These mixtures are in fact static, if you add to that the dynamic effect of seminal fluid dilution and disruption *in vivo* is it expected that we should relate these additional aspects of destruction to the studies of *in vitro* mixures. A lot of the studies that you refer to examined mixtures that were taken post-coitus. Is there any intention or any work on your part to study the effects of increasing periods of post-coitus interval before the collection is made on the impact of destruction of materials?

Martin: Swabs are collected every hour after intercourse; so samples are collected over a period of time post-coitus to see the effects.

Strolorow: I am expecting that there might be a more regular pattern of degradation and be able to plot some form of it. Yet the publications that have come from Davies and Willott indicate that what you expect to find in case work swabs in terms of locating factors that ought to have come from someone other than the victim, seems to be extremely erratic. Is that because we do not know what happened to those swabs between the time of assault and collection or is that something we might expect in studying casework materials opposed to laboratory specimens?

Martin: If you look at acid phosphatase the decreases in acid phosphatase looked at on those graphs that Davies and Willott drew up occurs fairly quickly at first and then begins to level off. However, on top of that then you've got to overlay the level on top of that then you've got to overlay the level of vaginal acid phosphatase which starts to be produced, it looks to me an hour or so after intercourse has taken place. The whole thing becomes very complex, because we don't know what the rate of degradation is of the pothenic acid phosphatase and what the rate of production is of the vaginal acid phosphatase. We only know it in isolation. When the two are together, how do we work it out. Now it may be the same for antigens as well or enzymes.

Question: In reference to antigens, would it be possible to identify antigens which are from someone other than the victim? The results of the study you referred to seem to show that there is very little relationship between the length of post-coital interval and finding the antigen that comes from the victim.

Martin: Samples need to be collected from at least 50 different donors to see if there is a general pattern.

Question: I think it is important to distinguish between loss of material and destruction of material The basic model that we have of loss of the material from the vagina is that there is two kinds of factors involved. One is mechanical loss that is just the mechanical drainage of semen from the vagina. The second is that the material which does not drain out is then subject to destructive processes in the vagina So that when you look at some of these studies that have been done you have to take into consideration that which is due to just drainage, mechanical drainage, and that which is due to destruction and not get the two confused. That material that is removed from the vagina simply is the results of drainage is going to involve all of the components of semen.

Sensabaugh: Obviously, we have to come up with some method or model for differentiating these types of losses. In any case, really it is a matter of scale isn't it We're talking about levels of acid phosphatase in semen that are a thousands times the level of the acid phosphatase in the vagina. You're talking about levels in the vagina that are going up by factor of 2 or 3 which is no where near the kind of scale that we are talking about when we are comparing semen in vaginal fluids. If you think about the way things would be the total amount of acid phosphatase it could be contributed to the vagina would be the amount that is in vaginal tissue to begin with so that would provide an upper limit or at least a reasonable approximation. Therefore, there should be an upper limit of the amount of acid phosphatase might go into the vagina into the vaginal fluids in one of these post-coital experiments. Do you see what I am saying?

Martin: Well I see, but I don't necessarily agree with you though. Why should it be only the level that is in the vaginal tissue at that time. Why can't it be actively produced?.

Sensabaugh: One source of it would be say the ephithelial layer of the vagina are breaking open and releasing all the acid phosphatase that it had. That would provide one kind of upper limit. The second would be conceivably due to stimulation of one kind or another, mechanical, presumably. You have active synthesis of acid phosphatase. Is that what you were referring to?

Martin: I am not saying it is that. All I do is give you the chance it happened.

Sensabaugh: Then the question is how much could you make and again I think what you've got so far really indicates the amounts of increase that you are getting are major increases of small amount of doublings or triplings of very small amounts which still leaves you with very small amounts.

Martin: The thing is it goes this way the two donors that we tried we normally get no acid phosphatase from the vaginal swabs from those two donors. So it may only look as though they are going up four times as much. That is four times as much on what I know is the base level there. The base level with around about one unit. Now we don't usually find anywhere near one unit per ml from our donors, but they were the first two we did and they were the ones that got drawn up We have found one since. I think we have only four donors and one of these was up in the level where we would normally be quite happy that we've got semen.

Sensabaugh: What level was that?

Martin. Around 80.

Sensabaugh: So that is in your sigma units.

Martin: Yes. That is historical. This work is all very much in its infancy at this time. The biochemistry at the post-coital vagina is obviously different from that of the resting position. We know very little about it.

Sensabaugh: In that regard, I seem to remember some years ago someone telling me, Dr. Brown, maybe you can give an answer better than I, that just the male in the course of the night undergoes periodic tumescence and relapse. Does the female also have these periodic periods that correspond more or less to a preorgasmic state? Is that so?

Brown: That is difficult to study because you don't have the same manifestation in the female that you do in the male, as far as an erection which can be observed. However, I will comment to further cloud

the issue, regarding the fluids that are found in the vaginal tract. One Dr. Sensabaugh eluded to is mechanical but the other is psychological. In the normal female, in both anticipation of and direct simulation during intercourse, there is swelling of the external genitalia and an increase in the volume of fluid that accumulates in the vagina Sexual assault, however, would evoke different emotional factors and there may not be this increase in fluid volume.

Sensabaugh. This comment regards the difference between your donor studies and your case studies. There are only a couple of reasons why that could be the case. I think the most likely reason is that your police surgeons are not collecting very good swabs.

Martin: That is exactly what I said. I think it is the only reason

Sensabaugh: I think that is an educational problem that all of us really face.

Martin: But we have tried in lectures.

Lee: A couple of years ago I questioned emergency room staffs from several hospitals and asked them how many swabs they collected. Six to ten swabs are collected, depending upon the hospital. We don't know which swab we will get, number one or number ten. So in one case I requested all ten. I did sperm counts on each and found drastic differences from swab to swab. At that time I was not analyzing for p30. I would estimate those results would probably be different also. I would suspect this is an international problem.

Lincoln: These last few days one of the things which has been concerning me is the fact that we talk about samples that we get, where we have semen in the vaginal fluid and we believe that degradation occurs and there is a loss through drainage. However, it is quite obvious there is a different rate of the destruction of the enzymes and of the ABO antigens. Even in the ABO system a different rate of destruction in a mixtures of semen and vaginal fluid occurs; the H disappears more quickly than the A.

Mudd: You have been talking about increased levels of acid phosphatase that might be found in the vagina area after intercourse in normal donors. You are talking about normal stimulation that might occur between two individuals. But in rape this is not normal at least on the part of the female and I wonder if it is possible to relate what happens in controlled circumstances in the laboratory to actually to what happens in the vagina in rape where the victim is not a willing party.

Martin: I think at the moment, we can't possibly do that but until we do the donor study we won't know what we are expecting and then we will have to do the casework study to see if it does fall in line. But it is going to take a hell of a long time.

SEXUAL ASSAULT EVIDENCE: VIEWPOINT OF THE DEFENSE EXPERT

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Over the last 10 to 15 years, there has been a considerable increase in the amount of information that can be obtained from the examination of swabs and stains associated with sexual offenses. As a result, not only is examination of these materials likely to produce useful evidence, but the expansion in the expertise is also reflected in the increased complexity of reports.

This greater potential means that there is more chance of producing evidence towards either convicting the guilty or exonerating the innocent. If the evidence from the body fluid examination is highly incriminating, particularly when the defendant is adamant that he is innocent, the defense will consider it important to have this evidence looked at by their own expert, especially when the sentence is likely to be severe if the accused is found guilty. The increased complexity of reports and sophistication of the expertise mean our legal colleagues need, more and more, the help of the appropriate expert to interpret the findings and assess their significance as far as the defendant is concerned. Moreover, the prosecution and defense inevitably view the case from different standpoints and this must on occasion influence the selection of scientific investigations performed and the manner in which they are reported.

In England, it is usual for the prosecution scientists, when they have completed their investigations, to report their findings in the form of a statement. This may mean, if prosecution or defense do not wish to call the witness to court, that the evidence presented in the statement is accepted into the trial without the actual appearance of the scientist and without elaboration or cross-examination. Such statements, containing the prosecution scientist's evidence, are made available to the defense lawyers usually fairly soon after they have been received by the prosecuting lawyers. The investigation has been carried out for the prosecution and although the defense receives copies of these statements, they cannot approach the scientist for any discussion or help. What kind of advice is sought by the defense lawyer on behalf of his client? In my experience, the investigations can be divided into the following broad categories:

1. **Material may be tested which has not been investigated by the prosecution.** This may be because it is of interest to the defense but not the prosecution, the material may not have been available to the prosecution or they may have elected not to test it.

2. **Retesting of material to check results obtained by the prosecution.** This may be requested when testing of material by the prosecution has not produced conclusive results, in which case the defense may decide to ask their own expert to perform tests on any remaining material. Alternatively, it may be that results have been obtained which are very damning for the defendant or are not consistent with his version of the events.

3. **Testing for further polymorphisms which have not already been investigated by the prosecution.** Results from such testing could be helpful to the defense but, alternatively, they can provide additional evidence in favor of the prosecution case.

4. **Interpretation and evaluation of scientific evidence.** The defense may require an explanation of the prosecution scientist's results, including comments on the reliability, interpretations, implications and conclusions, but may not require additional laboratory work.

1. Testing material which has not been investigated by the prosecution

Inevitably there must, at some stage, be selection of the material which is to be subjected to testing. This may be a selection which takes place before submission of materials to the laboratory or it may occur in the laboratory; but wherever it occurs, it can mean that certain items have not been tested and the defense may decide that it could be in their interest to have them investigated. Alternatively, it may be that there are materials of interest to the defense of which the prosecution is not aware. Such situations can arise from the defendant's own explanation of the alleged events.

In the first example case involving two males in a public toilet, one of the accused men, of high intelligence, realized the importance of having his own investigation to produce evidence in support of his denial of the allegations.

The accused was examined by the police doctor, but

the prosecution decided they were not interested in the penile swab and swabs taken from the hands of the accused and so he retained these materials himself and they were submitted to me via the defending lawyers for examination. Also submitted for examination were a pair of underpants which the accused said he was wearing at the time in question and on which, if the prosecution allegations were correct, it seemed likely that there might be semen. Examination of the swabs and pants in our laboratory, on behalf of the defense, failed to demonstrate any semen and this was in support of the man's denial of the alleged offenses.

In a second example case, the testing of an additional person allowed an alternative interpretation of the results as illustrated in Table 1. The victim was a 63-year-old woman and the 17-year-old accused admitted that he was in her apartment with intent to steal, but pleaded not guilty to rape of this lady. There had been two other recent incidents of rape in the same apartment block, one resulting in the murder of the victim, and there was some concern, however remote the possibility, that the 63-year-old victim had imagined the alleged attack by the intruding defendant. The original report by the prosecution scientist referred only to the presence of semen on two vaginal swabs, a piece of carpet and the victim's skirt. A later report gave results of ABO grouping on the defendant, victim and on the various stains, as set out in Table 1. Reactions for A secretor were found on the vaginal swabs while the reactions obtained on the carpet were, according to the prosecution report, consistent with there being a mixture of semen from the accused and vaginal material from the victim. The defense requested samples from the victim's husband for testing. Although he said he had last had intercourse with the victim about two weeks prior to the alleged offense, he was not sure of the date. Tests on this man showed him to be group O secretor, the same as the accused. Also, our testing of the vaginal swabs demonstrated A but no H activity while on the carpet both A and H activity were detectable. This, in fact, was in agreement with the prosecution scientist's findings although it was not apparent from the statements. Thus the defense examination showed that there was no evidence for the semen on the swabs being group O secretor and, moreover, if the reactions obtained on the carpet stain were due to a mixture of O secretor semen and A secretor vaginal material, this semen could, from the blood group point of view, have originated equally well from the victim's husband as from the accused.

A not uncommon situation arises where the accused has declined, as is his right, to donate the necessary blood and saliva samples for the prosecution investigation. This can result in the defense having their own expert test the appropriate samples at a later date. A slight variation of this situation occurred in a case referred to my laboratory where the accused man and the victim of rape had each produced the necessary blood and saliva samples after the alleged offense and results obtained from these were used to interpret saliva groupings obtained on a cigarette (see Table 2). However, the only semen found on the various items investigated was on the victim's coat and the prosecution scientist stated categorically that no sperm were found in this semen stain, thus indicating that the semen was from an oligospermic or azoospermic male. No grouping results were reported. There appears to have been some informal comment by the police directly to the accused that they would like to test a sample of his semen. The defense solicitors, hearing of this, were somewhat puzzled by what was to them an unusual request and consulted me. The defendant then produced a semen sample for me to test and this showed that he had a normal sperm count. A portion of this sample was offered to the prosecution who confirmed the results. The man was acquitted of the offense by the court.

Table 1. A DEFENSE EXAMINATION WHICH INVOLVED TESTING A SECOND MAN WHO HAD NOT BEEN TESTED BY THE PROSECUTION

		Prosecution Results	*Defense Examination*
Victim		A Secretor	
Accused		O Secretor	
Vaginal Swabs	Semen	A Secretor	A activity, no H
Carpet	Vaginal and seminal material	Reactions consistent with mixture of semen from accused and victim's vaginal material	A and H activity
Husband			O Secretor

Table 2. A CASE INVOLVING AN EXAMINATION OF THE ACCUSED MAN'S SEMEN WHICH HAD NOT BEEN SUBMITTED TO THE PROSECUTION

	Prosecution Results	*Defense Examination*
Victim	Le^{b+}	
Accused	Le^{b+}	Normal sperm count
Cigarette	Saliva–Le^{b+} reactions	
Blouse, three vaginal swabs, debris and swabs from scene	No semen	
Victim's coat	Semen—No spermatozoa detected	

2. Retesting of material to check results obtained by by the prosecution

When one thinks of a defense examination, this is the kind of investigation which usually comes to mind. When the charge is very serious, the scientific evidence very damning and because juries may be warned in sexual offense cases that it may be dangerous to convict on the uncorroborated testimony of the victim, it is not surprising that defense lawyers request that the prosecution results be checked by an independent expert. This is even more likely to happen if the accused is strenuously denying the charges against him.

In this kind of situation, the defense expert has, to some extent, a difficult task. Generally, because of the legal system in our country, the defense examination is not carried out until many months after the alleged offense and examination by the prosecution scientist. This means that some blood-group polymorphisms which would be detectable in fresher stains will have lost some or even all of their activity. The defense investigator must be very cautious in the interpretation of any results he obtains when these do not agree with those of the prosecution scientist. This is particularly true when the defense expert fails to detect a blood-group polymorphism which had previously been shown to be present. Hence, the advantage of an early defense examination.

A further disadvantage arises from the fact that some stain material will have been used in the first examination. This not only reduces the quantity available, but also the quality of that remaining may be inferior since it may not be the most active part of the stain. However, the detection of a blood group which had not been detected in an earlier prosecution examination is likely to be a valid finding.

The defense expert does have some factors in his favor. The large numbers of cases and exhibits referred to prosecution scientists will allow them to gain considerable experience, but it may inevitably curtail the time which can be given to the examination of an individual exhibit. The defense scientist may be more fortunate because the number of cases referred may be smaller and usually I find I am asked to direct my attention to particular exhibits only. This means it is often possible to spend more time, and in my situation my own time, on a much smaller number of problems. Furthermore, I am usually aware of the prosecution results, and this means that if my results differ from those of the prosecution, provided there is sufficient material, I can perform repeat tests to give added confidence to my findings.

Also, knowledge of the results already obtained and the fact that one is being asked to look at the case from a different point of view may mean that one can make some useful comment even on results which the prosecution scientist might consider inconclusive or on materials considered not worth testing. Frequently the defense expert is asked to carry out repeat tests in order to attempt to group stains which the prosecution has found or considered inadequate or on which their results have not been conclusive. A semen stain which remains ungrouped can, of course, originate from any male, but it has the potential of producing evidence which would exonerate the defendant. Moreover, seen from the defense point of view, the evidence of semen of an unknown group, presented to a jury by a good prosecuting lawyer, can sound as damning as semen which has been grouped and shown to be the same type as that of the defendant!

The example case summarized in Table 3 illustrates that even when a defense examination fails to produce a completely conclusive grouping result, enough information may be gained to be of interest to the defense lawyers. In this rape case, semen had been demonstrated by the prosecution scientist on the panties, two vaginal swabs and two anal swabs taken from the victim, but grouping tests were unsuccessful. At the time of this examination, samples were obtained from the victim and the accused, and grouping results showed them to be group O secretor and nonsecretor, respectively. Two months later, samples taken from the victim's boyfriend demonstrated that he was an AB secretor. While the various semen stains in this case remained ungrouped, the defense considered that the possibility remained that their client could be exonerated by this evidence. My investigations for the defense did not detect any blood group activity on the anal swabs or the panties, but trace amounts of H activity were detected on the vaginal swabs. This could have originated from the victim but did not rule out the possibility of the semen originating from the accused. However, no evidence for A or B activity, which would have suggested the presence of semen from the victim's boyfriend or someone other than the defendant, was

Table 3. RE-TESTING MATERIAL ON WHICH THE PROSECUTION RESULTS WERE UNSUCCESSFUL

Prosecution report, March	
Victim	O Secretor
Accused	Nonsecretor
Panties, anal and vaginal swabs	Semen—Grouping unsuccessful
Prosecution report, May	
Boyfriend	AB Secretor
Defense examination	Small amounts of H activity on vaginal swabs

detected. Thus the defense was satisfied that this part of the prosecution evidence had been checked and, moreover, examination by their own expert did not produce any results to support the defendant's denial of the alleged offense.

A further example case involving a similar situation which I find is of common occurrence is summarized in Table 4. Here, the prosecution scientist found that the grouping results were inconclusive on two swabs which he tested, but grouping was not attempted on a further two. My examination for the defense of all four swabs produced inconclusive results on the same two which had produced inconclusive results in the hands of the prosecution; but on the two previously untested swabs I was able to demonstrate a small amount of B activity. This did not exclude the defendant as a possible source of the semen since the victim was group B secretor and the accused group B nonsecretor. The defense examination had not produced any evidence to suggest that the semen had originated from anyone other than the accused. The fact that these swabs had been tested by their own expert, even though the results obtained did not help to exonerate the defendant, was useful to them in the defense of their client.

Table 4. TESTING MATERIAL WHICH HAS BEEN SUBMITTED TO THE PROSECUTION BUT HAS NOT BEEN TESTED BY THEM

	Prosecution results	*Defense results*
Victim	B Secretor	
Accused	B Nonsecretor	
Four vaginal swabs, semen		
Swabs 1 and 2	Grouping inconclusive	Grouping inconclusive
Swabs 3 and 4	Grouping not attempted	Small amount of B activity

In such situations, a client maintaining his innocence and insisting that all possible avenues of investigation are exploited can, at least to some extent, be appeased by his lawyer engaging the appropriate expert even when the results do nothing to support his claim of innocence!

Furthermore, the legal situation in England at the present time is such that the defense can have an investigation carried out knowing fully well that it is not likely to have to divulge the outcome to the court. Hence, they are not at any greater disadvantage if the results are unfavorable to their client!

3. Testing for further polymorphic systems which have not been investigated by the prosecution

It may be appropriate from the defense point of view to carry out testing for polymorphisms which have not been attempted by the prosecution. For instance, if the defendant is adamant that a particular stain could not have come from himself, but the grouping obtained by the prosecution scientist shows nothing against such an occurrence, tests for further polymorphic systems could be performed by the defense expert because these might show that the stain could not have originated from the individual in question. Moreover, the limited quantity of material and the age of the stains mean that it is often more profitable to test for further polymorphisms rather than to retest to check any results already obtained by the prosecution.

In the example case summarized in Table 5, an Asian man was accused of indecent assault. The prosecution scientist considered that there was insufficient semen on the anal swab to attempt grouping tests, but on the other exhibit submitted, the victim's underpants, he found semen which he was able to group as PGM 1, the same group as the accused and 1 in 2 of British Asians. Four months after the alleged offense, the defense lawyers asked if I could perform any further grouping which might exonerate the defendant, especially as the blood-grouping evidence obtained by the prosecution did not provide particularly strong evidence towards the semen originating from their client. Successful ABO grouping tests were obtained on the semen-stained underpants, but they showed that the semen was from a group O secretor individual, the same group as the defendant. While in this case the result did not help to exonerate the defendant, the fact that this further evidence had been produced helped to console the defense lawyers in their efforts to do everything possible for their client and to test the reliability and significance of the prosecution evidence.

Table 5. TESTING MATERIAL FOR ADDITIONAL POLYMORPHISMS NOT ATTEMPTED BY THE PROSECUTION

Prosecution results		
Victim		A, PGM2-1
Accused		O Secretor, PGM 1
Anal swab	Semen	Insufficient to attempt grouping
Victim's underpants	Semen	PGM1 (1 in 2 British Asians)
Defense results (4 months later)		
Victim's underpants		Group O secretor reactions

4. Interpretation, evaluation and advice on results

Of the various kinds of work requested by the defense lawyers, this is probably the most common and can be of great importance. There are many aspects. Because of the very specialized nature of the expertise involved, lawyers quite understandably need professional advice and help concerning the implication and reliability of results. They may need interpretation of the findings, advice on their value and often an explanation of the meaning of such specialized terms as "PGM," "GLO," "ABO secretor." No matter how experienced in his own profession, a lawyer trying to understand reports involving these terms can be baffled (see Table 6). Explaining to a lawyer that "PGM2 -1" means that two factors, PGM1 and PGM2, are present, whereas in the Gm system, "Gm1, -2" indicates that the 1 factor has been found to be present but the 2 factor is absent because of the " -" sign can be difficult, particularly as on another occasion we may confront them with PGM2-1+ and expect them to understand that this means two factors, PGM2- and PGM1+, are present!

Table 6. EXAMPLES OF SCIENTIFIC TERMINOLOGY WHICH MAY NEED EXPLANATION TO THE NON-EXPERT

GLO 2-1
PGM 2-1
Gm 1, -2
PGM 2-1+

The need for defense lawyers to understand adequately the scientific evidence is appreciated, but in order to achieve this, it will be more and more necessary for them to seek expert advice. In my experience, it is not unusual, even when a lawyer thinks he has understood a report, for this not to be so to varying extents!

Questions posed by defense lawyers tend to be along the lines of:

Could or should more tests have been done?

Should not the evidence have been more conclusive?

Why were certain tests not done or not conclusive?

How reliable are the tests and what weight can be put on the results?

Are these the usual tests carried out on such material?

For how long is semen detectable?

In England, inevitably the prosecution statement has been prepared with the prosecution rather than the defense objectives in mind and with only the information known to the prosecution. Hence, an assessment of the evidence from the defense point of view may produce comments which differ significantly from those of the prosecution.

The example case shown in Table 7 illustrates that although the results and comments reported by the prosecution scientist may be correct, it may be relevant to know what remains unsaid! In this case, there was little scientific evidence connecting the accused with the victim, but the finding of semen which could have come from the accused on the victim's nightdress might have appeared to provide the link between the two. Quite correctly, the prosecution scientist had concluded that the group A secretor reactions obtained could be due to vaginal debris from the victim (A secretor) mixed with semen of group A secretor, O secretor or nonsecretor and therefore could have come from the accused (O nonsecretor). What the scientist had not pointed out was that these groups constitute about 90 percent of the British population, so it was not unexpected or very significant that the accused had not been excluded from being a possible donor of the semen!

Table 7. AN EXAMPLE OF THE IMPORTANCE OF WHAT IS LEFT UNSAID IN A REPORT

Victim		A secretor
Accused		O nonsecretor
Vaginal, anal and thigh swabs; victim's panties	No semen	
Penile swab	No semen or vaginal debris	
Victim's nightdress	Semen and possible vaginal cells	A secretor reactions
	Could be due to vaginal debris from victim mixed with semen of group A secretor, O secretor or non-secretor. The accused is a non-secretor.	

Sometimes defense lawyers ask if the explanation given by the prosecution scientist is the only interpretation that could be placed on the results. Table 8 shows results illustrating such a case. The man accused of rape was group A secretor and the victim group B, secretor status unknown. Semen was found on two vaginal swabs and according to the prosecution scientist's statement, group AB reactions were obtained on these. The only explanation offered was that "since the victim was group B, this reaction could be due to the victim's own secretions, together with semen of group A or AB. The accused is group A." This interpretation assumes that the victim is a secretor, if not, the B reaction could not have come from her and this man would have been excluded as a possible source of the semen. Other interpretations, involving the possibility that no blood-group activity from the victim was detectable which

Table 8. AN EXAMPLE CASE WHERE THERE ARE ALTERNATIVE EXPLANATIONS FOR THE PROSECUTION FINDINGS WHICH HAVE NOT BEEN OFFERED BY THE PROSECUTION

Victim		B (secretor status unknown)
Accused		A secretor
Two vaginal swabs	Semen	AB reactions
	"Since the victim is group B, this reaction could be due to the victim's own secretions together with semen of group A or AB The accused is group A "	

would exclude the accused as a possible donor of the semen, were not mentioned in the scientist's statement, but were relevant to the defense lawyer's question.

In a recent case submitted to my laboratory, only one possible explanation was given for the A secretor reactions obtained on a semen-stained vaginal swab. The victim was group A secretor, the accused O secretor, and the scientist explained that the reactions obtained could have originated solely from the secretions of the victim. The alternative possibility, that these reactions were due solely to the semen or to semen of group A secretor mixed with activity from the victim, was not mentioned, but either explanation would have exonerated the accused.

The fact that it is not possible to determine which fluid is giving rise to which particular blood-group activity causes some confusion and frequently has to be explained to our legal colleagues.

Interpretation by the prosecution of the findings implicating the accused in terms of the alleged offense without consideration of alternative explanations is not uncommon in the casework presented to us by defense lawyers. A further example of lawyers asking if there is any other possible explanation for the prosecution findings is shown by the case summarized in Table 9. In this case, semen was detected on vaginal swabs and the victim's panties, but the scientist found them impossible to group. However, several semen stains were found on the accused man's underpants and one of these was grouped and shown to give group B reactions. The accused was group A nonsecretor. The scientist said that this could be due to semen from the accused mixed with a body fluid from a person of group B and pointed out that the victim was group B. No saliva was found on the victim's external vaginal swab, no semen on her mouth swab or in her saliva sample. The defense lawyers requested elucidation of this report and also asked if this was the only possible explanation of these findings. There were a number of points which could be made. One alternative explanation was that the reactions obtained were originating solely from semen, a group B semen. For this interpretation, one had to suggest the presence of semen from some other male being present on these underpants (had some other male been wearing them or had another male ejaculated in them?). In this respect the lawyers were mindful of the fact that their client was not likely to admit readily to the latter of these possibilities. Other questions which needed answering included the lack of mention of any evidence for the presence of another fluid in the semen stain, the lack of any testing for Le^a substance which might have helped since the accused was a nonsecretor, and also the apparent failure to find any trace of the A activity expected in an A nonsecretor semen. One presumes that if the accused had been group B, the results obtained would have been interpreted as the activity of a group B semen and no alternative explanation sought, instead of which this usual explanation had not been mentioned!

Table 9. AN EXAMPLE CASE WHERE THE PROSECUTION HAVE INTERPRETED THEIR RESULTS ONLY IN THE MANNER WHICH IMPLICATES THE ACCUSED

Victim		B secretor
Accused		A nonsecretor
Vaginal swabs and victim's panties	Semen	Unable to group
Underpants of accused	Several semen stains	One stain group B. Could be due to semen from accused mixed with body fluid from group B person. The victim is group B.

Occasionally, not only is advice needed on the value and reliability of the evidence, but snippets of knowledge gained by members of the legal profession encourage them to raise their own questions. In a recent case where paper tissues from the inside of the victim's panties gave reactions for A secretor Pep A 2-1 (the accused man's groups), with indications of B activity (the victim's group), the lawyers were eager to have an assessment of what appeared to be strong evidence against their client. They needed an opinion concerning the reliability of the Pep A grouping and the frequency of these groups. Also, they were aware that the B antigen could be acquired, albeit rarely, and they were concerned whether A could also be acquired! Although they accepted the frequency of 3 percent for British Negroes being type Pep A 2-1, they queried whether the fact that their client was an American might have some influence on this aspect.

Another recent case also illustrates the need for

lawyers to be able to appreciate the implications and the value of the scientific evidence. Semen of group B secretor was found on the vaginal swab from a group O victim. The accused was group B secretor but was black and from North Africa. Although only 6 percent of British men would be of group B secretor, this piece of evidence lost some of its value if looked at from the point of view that the chance of a wrongly accused black North African being of the group B secretor was in the region of 20 percent (i.e , a 1 in 5 chance).

It is still possible to see cases where the victim's group has not been referred to in the statement of the scientist. The results from such a case are shown in Table 10. The scientist concluded that there was semen from a group A male on two vaginal swabs, but in his statement there was no mention of the ABO group of the female victim. One assumes that this was an omission from the statement since the victim's PGM group had been determined, was given in the statement and was taken into account in interpretation of the PGM results obtained on these swabs. However, the defense lawyer would be failing in his duty if he did not ascertain by cross-examination the situation concerning the ABO group of the victim. Moreover, such an omission is unlikely to be noticed without the help of an expert.

Table 10. AN EXAMPLE CASE IN WHICH VICTIM'S GROUP IS NOT GIVEN IN THE REPORT

Victim		PGM2-1+
Accused 1	A secretor	PGM1+1-
Accused 2	O	
Accused 3	B	

Vaginal swabs: Semen from group A, also PGM2-1+1- reactions. PGM2-1+ could have come from victim but not the PGM1- reaction The semen could be from a man of type PGM2 1-, 1+1- or 1 .

The increasing complexity of reports is illustrated by the results shown in Table 11. In this case, the prosecution scientist tested blood and saliva samples from the victim and seven accused, but did not test samples from an eighth accused who was arrested at a later date. I was asked to test this eighth person and to give assistance to the defense in the interpretation of the evidence. Not surprisingly, the defense lawyers found the prosecution's eleven-page report of the biological evidence concerning the seven accused and involving some 120 items which had been investigated somewhat indigestible and requested a review of the serological results and explanation including the new information obtained from testing the eighth accused person. As well as adding this information and attempting to clarify the evidence, it also became apparent that there were additional comments which could be made concerning the skirt On this item the prosecution scientist found a mixture of blood and semen which gave reactions for AB, PGM1+. In commenting on this finding the prosecution scientist stated that this could be due to a mixture of the victim's blood and semen from accused 4, but that semen from accused 2 and 3 could also be present. However these last two individuals were of groups PGM2+1+ and 1+1−, respectively, and the prosecution scientist seems to have made an error in his comment that their semen could also be present in the stain which he grouped as PGM1+. Moreover, the only explanation given for the grouping result obtained on the skirt was that which fitted with the groups of the individuals presented for testing. Alternative explanations involving group AB or B semen were not mentioned. Maybe the reactions obtained allowed the scientist to rule out these possibilities, but as far as the evidence produced in the statement was concerned, the defense lawyers were justified in asking if these results could not be explained in some other way.

Table 11. THE INCREASED COMPLEXITY OF REPORTS AND INCORRECT INTERPRETATION OF RESULTS BY THE PROSECUTION SCIENTIST

		ABO	*PGM*
Victim		B	1+
Accused 1		O	1+1-
2		A	2+1+
3		A	1+1-
4		A	1+
5		O	1+
6		O	1+1-
7		O	1+
Skirt	Blood/Semen	AB	1+
Blanket	Blood/Semen	AB	
Bedspread	Blood	A	
Sheet 1	Semen		1+
Sheet 1	Semen		2+1+
Swab from victim	Semen		
Sheet 2	Semen		
Sheet 3	Blood/Semen		
Sheet 3	Blood		
Sheet 4	Semen		1+
Sheet 5	Blood		
Defense examination			
Accused 8		A	2+1+

Another example of a case which illustrates the complexity of reports facing our legal colleagues is outlined in Table 12. It can be seen that two victims and four accused were involved. In addition to the

ABO and secretor typing as shown in the table, the extensive report from the prosecution scientist also dealt with bloodstains on which PGM and Hp groupings had been performed. The grouping results obtained on each of 18 exhibits were carefully described including the fact that on many items there was the possibility of mixtures of fluids and of semen from more than one of the accused. The prosecution named those of the accused who could not be ruled out as possible donors of the various stains but did not name those of the accused who were excluded. In this case, the many alternative explanations including the groups of semen which could be masked by the reactions obtained were given, probably because they involved the various accused men who had been tested. This of course resulted in a very complex report. In such a situation it is, in my experience, often very helpful to lawyers if the results can be summarized in a table, and this often clarifies a pattern of exactly what the blood group evidence is showing concerning the various individuals and items which have been tested. Such tabulation was not attempted by the scientist in this particular case, but is becoming a common feature of many of the reports from prosecution scientists in England.

Table 12. EXAMPLE OF A COMPLEX REPORT INVOLVING POSSIBLE MIXTURES OF FLUIDS, A NUMBER OF ACCUSED AND TWO VICTIMS

Victim 1		O nonsecretor
Victim 2		A secretor
Accused 1		O secretor
2		A nonsecretor
3		O secretor
4		A secretor
Panties (victim 1)	Semen	O secretor
Panties (victim 2)	Semen	A secretor
Underpants (accused 1)	Semen	A secretor
Underpants (accused 3)	Semen	A secretor
Underpants (accused 4)	Semen	A secretor
Sheet	Semen	O secretor

In my experience, interpretation of results where there can be mixtures of body fluids and also possible masking effects of the blood grouping activity is an area in which lawyers often need expert advice. This is particularly so, not only because of the complicated nature of the interpretation, but also because, in my experience, possible alternative explanations may be mentioned if they are relevant to the suspects tested, but not be given where only one suspect is named or if they do not involve the consideration of the named suspect.

SUMMARY

The defense examination can, and often does, involve retesting of materials examined by the prosecution scientists, but I hope that from the examples given above, it will be clear that this is only one aspect of a much wider field of work which is requested by defense lawyers. Moreover, much of the work, it seems to me, concerns not only the problems of the defense lawyer, but could equally well apply to the prosecuting lawyer who needs advice on the reliability, the implications, the relevance and even some help with the understanding of this specialized expertise if he is to use it to its full value in the court.

My experience is that defense investigations very seldom show that errors have occurred in the testing performed by the prosecution scientist, but that further tests and possible alternative interpretation and evaluation of the prosecution results may be appropriate when the case is viewed from the defense standpoint.

The marked increase in the number of cases referred to my laboratory by defense lawyers over the past few years would appear to support the idea that such a service is not only needed by the legal profession, but is increasingly used.

SECTION II—SHORT PRESENTATION ABSTRACTS

DEVELOPING AN EFFECTIVE SEXUAL ASSAULT EVIDENCE KIT PROGRAM

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Sexual assault cases have the potential for having large amounts of significant evidence. In this unique situation, the investigator or police officer is not allowed to collect the best evidence himself or, in most cases, even to be present when it is collected. The collection of evidence can only be done by health-care personnel during the medical examination of the victim. In order for the crime laboratory to receive the best possible evidence, it is necessary to:

1. Provide a well-designed, easy to use collection kit to the medical personnel.
2. Educate the medical personnel in the correct use of the kit, explaining the reasons for collecting various evidence and the importance of the chain of custody.
3. Train police officers in the proper preservation of the evidence, stressing prompt submission to the laboratory.

When the above enumerated conditions have been achieved, a significant increase in the evidential value of specimens is achieved.

THE NORTH CAROLINA RAPE KIT PROGRAM

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In November, 1979, a statewide program for the collection of evidence from victims of sexual assault was initiated by the serology section of the North Carolina State Bureau of Investigation (NCSBI). This program included the design and assembly of a standardized "rape kit," distribution of these kits to hospitals and preparation of audio-visual aids to demonstrate the proper procedures involved in the collection, preservation and analysis of such evidence. This educational program was directed at emergency room and other hospital personnel in approximately 125 hospitals during 20 training sessions conducted throughout the state.

The effectiveness of this program was evaluated by the N.C. Council on the Status of Women via telephone and written surveys; members of the serology section evaluated each kit submitted and notification of improper usage and appraisal of corrective measures needed were made to the hospitals by the rape kit program coordinator.

Legislation was enacted in North Carolina in July, 1981, which specifies the use of NCSBI-approved

TYPING RESULTS ON SEXUAL ASSAULT LABORATORY OF CRIMINALISTIC

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Approximately 1,300 sexual assault kits have been examined in our laboratory from 1977 to the present.

No semen was found on evidence in 492 kits (38 percent). ABO typing was attempted on evidence from 821 of the semen positive kits with the following results:

In 474 cases (58 percent) the ABO type found was that of the victim.

In 173 cases (21 percent) foreign A and/or B activity was found.

In 138 cases (17 percent) no ABO activity was found.

In 36 cases (4 percent) H(O) activity only was found, though the victim was type A or B.

The secretor status was determined by saliva swab analysis on 741 victims. We classified 66 non-secretors (22 percent) among the 373 individuals of type A, B or AB and we classified 92 non-secretors (33 percent) in the group of 368 type O individuals. These results suggest that dried swabs are not reliable samples for the determination of the secretor status of the victim. Phosphoglucomutase (PGM_1) analysis was attempted on evidence from 533 of the semen positive kits. Foreign PGM activity was found in 52 cases (10 percent). The table below of PGM types found illustrates that the victim's PGM type is present in the majority of the cases.

PGM type found on evidence	*PGM type of victim*		
	1	*2-1*	*2*
1	152	—	—
2-1	45	140	7
2	—	—	15
No activity	107	59	8

A PROGRAM FOR RAPID COLLECTION OF SEXUAL ASSAULT EVIDENCE IN RAPE-HOMICIDE CASES

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Timely collection is a critical factor in preserving seminal evidence from vaginal samples for typing. For example, a recent study (Price *et al.* J. For. Sci. Soc. 16:29, 1976) indicated that seminal PGM is viable for no more than six hours postcoitus. In the past in Los Angeles County, post-mortem sexual assault evidence in suspected rape-homicide cases has been collected following transportation to the coroner's facility, occasionally several days later. This delay has compromised the typeability, as well as the detectability, of semen contained in those samples. Recent reorganization within the Los Angeles County Chief Medical Examiner-Coroner's Office has permitted the response of criminalists to scenes of homicides. These criminalists are trained and equipped to collect sexual assault evidence at the homicide scene in appropriate cases, avoiding the additional degradation caused by time spent in transporting the victim. Samples are dried and stored frozen until analyzed. Portable swab drying boxes with forced air flow are being investigated as a means of shortening drying time to further minimize degradation. It is anticipated that this program will make PGM typing possible on post-mortem sexual assault samples in those cases in which the body is discovered within a few hours of death.

STATISTICS REGARDING SEXUAL ASSAULT CASES

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Testimony in regard to sexual assault cases often leads the expert witness into the area of questions in regard to the frequency and correlation of certain laboratory findings. Due to the small amount of published data in this area, our laboratory undertook compilation of testing results for sexual assault cases over a 6-year period of time in Shelby County, Tennessee.

The data used were established by a manual review of analysis work sheets and categorization of testing results. Tallies were then made for each year in order to obtain frequencies and percentages.

Areas included in the study were: (1) frequency of the different types of alleged sexual assault, (2) results of secretor status determination, (3) correlation of the *l*-tartrate inhibitable acid phosphatase test and micro-

scopic findings of spermatozoa in each type of sexual assault, (4) results of testing external genital wipes for the presence of acid phosphatase, (5) frequency of detection of foreign blood-group substances in each type of assault, (6) frequency of microscopic detection of motile spermatozoa, (7) comparison of microscopic examination for spermatozoa by the initial examiner and the forensic laboratory and (8) frequency of detection of saliva and/or foreign blood-group substances from breast swabs.

After initial review of these data, we requested that our evidence collection agency also submit information concerning the length of time from the alleged sexual assault and from the last consensual sexual intercourse until the time of evidence collection. These time frames have now been incorporated into our statistics

The information gathered from this project has proven useful not only as a data base but also as a tool in establishing testing protocols. Additional data continue to be added as they become available.

THE LABORATORY REPORT IN SEXUAL ASSAULT CASES

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Little consideration has been given in the literature to the manner in which laboratory findings should be reported to the ultimate users of the laboratory services. In view of the complexities of interpretation inherent in sexual assault evidence, perhaps more concern regarding our role in reporting our results is in order. Many forensic scientists regard their written reports merely as a means of recording analytical results, rather than as vehicles for conveying their conclusions about their results. Persons who hold this view generally consider that the courtroom is the appropriate place for interpretation, despite the fact that many critical decisions about the investigation or disposition of a case may be made well in advance of trial on the basis of a written report by police officers or attorneys without benefit of any direct contact with the forensic scientist. An alternative view of the function of the laboratory report is that it should communicate to its reader both the analytical results and the interpretations and conclusions of the analyst, conveying the essence of what the expert would say if asked for his opinion in court. A useful philosophical approach to preparing this type of report in a sexual assault case is for the analyst to ask himself three questions: (1) Considering only the data derived from the evidence and the victim's reference specimens (disregarding any typing data from the suspect), which of my typing results can I attribute to the donor of the semen in this case? (2) Comparing the suspect's type(s) with type(s) attributable to the semen donor, can I exclude the suspect as the donor? (3) If he is not excluded, what proportion of the population shares his type(s) and should also be included as potential sources of the semen in this case? The answers to these questions may then be incorporated in a conclusionary statement which will serve to place the typing results, reported in tabular form, in their proper perspective. This style of reporting, by making clear both the value and the limitations of the analytical results, allows the user of the report to make intelligent assessments about the case with a minimum of confusion.

EFFECTIVE PRESENTATION OF SEXUAL ASSAULT EVIDENCE IN THE COURTROOM

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One of the primary objectives in collecting and analyzing sexual assault evidence is the hope that it will prove useful in the courtroom.

Sexual assault laboratory data by their nature are often complex and complicated. The "question and answer" format required for testimony and the generally elementary level of scientific background possessed by the average juror serve to frustrate effective evidence

presentation Therefore, planning, preparation and innovation are critical in making the scientific testimony understandable and understood.

Several steps to remedy this problem are suggested:

1. The scientific witness should view his or her role to be analogous to that of an educator
2. The witness should insist that the attorney requesting the testimony gain an understanding of the scientific aspects of the evidence before framing his or her questions.
3. The witness should participate actively in structuring the presentation of the evidence, including the framing of questions.
4. The witness should, with few exceptions, be available to "educate" the "opposing" attorney.
5. Innovative "teaching" methods should be considered, for example:
   (a) Direct courtroom microscopic demonstration of spermatozoa,
   (b) Photographic slides and prints of spermatozoa, and other tests and testing procedures,
   (c) Drawings and
   (d) Charts, graphs, tables and summaries of scientific data.

EVIDENTIARY COLLECTION AND EXAMINATION OF MALE SEXUAL ASSAULT VICTIMS

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The number of examinations of males for evidence of sexual assault has increased in Dallas County in the past year. In 1982, 42 males were examined and 12 have been examined during the first three months of 1983. The victims ranged in age from 9 months to 55 years with 31 (45 percent) under the age of 10. Only 15 (22 percent) were in jail at the time of assault. Evidence of injury, documented by the examining physician, was observed in 40 (58 percent) of the cases; of these, 18 showed anal injury. Laboratory analysis using quantitative acid phosphatase and/or microscopic examination for spermatozoa was positive in 16 percent of the cases by examination of perianal, anal or oral swabs and smears.

THE RELATIONSHIP OF PROSTATIC ACID PHOSPHATASE AND PROSTATE ANTIGEN IN LIQUID SEMEN SPECIMENS

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Prostatic acid phosphatase (PAP) has been used quantitatively to identify suspect stains as seminal in origin in the absence of spermatozoa and may be supported by two other chemical tests such as spermine and choline. Another seminal component that is also prostatic in origin has been identified and termed E1 by Li and Shulman (Int. J. Fertil., 16:87, 1971); termed γ seminoprotein (γ-Sm) by Koyanagi *et al.* (Jap. J. Leg. Med., 26:78, 1972) and p30 by Sensabaugh (J. For. Sci., 23:106, 1978), both of whom suggested its forensic application; and reported extensively in the medical literature as prostate antigen (PA) by Wang *et al.* (Invest. Urol., 17:159, 1979) who have suggested its use as a marker for prostatic carcinoma when found in serum.

We were interested in analyzing a large number of semen specimens to establish our own data base on seminal components and now report on the relationship between PAP and PA as determined from our study. Consequently, we collected semen specimens from 191 individuals and quantitated PAP using the substrate p-nitrophenylphosphate and PA by radial immunodiffusion. We found that both PAP and PA levels were distributed normally in these semen specimens. The mean level of PAP expressed as units/ml of semen was 467 ± 255 with a range of 10 to 1400. The levels of PA in these specimens ranged from 0.3 to 4.2 mg/ml with a mean of 1.2 ± 0.4 mg/ml. Although there was a positive relationship between PAP and PA (correlation of $+0.64$), this was not considered significant largely due to the wide variation in PAP levels.

SEMINAL ACID PHOSPHATASE (AP), P30 AND ABH LEVELS

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If the quantity of seminal fluid on a stain could be determined, then, theoretically, the sample could be diluted to a point where vaginal ABH activity would cease and any remaining activity could be attributed to the seminal fluid. To test this hypothesis we analyzed 25 semen samples.

Serial dilutions ranging from 1/100 to 1/1000 were prepared for each semen sample and the AP and p30 levels were compared. To determine the ABH levels, absorption-inhibition (AI) was performed on semen dilutions ranging from 1/10 to 1/9000, while absorption-elution (AE) was performed on threads prepared from semen dilutions ranging from 1/10 to 1/900.

The AP levels showed a large variation. As an example, the lowest level of the 1/100 dilutions had the same value as the highest at 1/900. The counterimmunoelectrophoresis of p30 showed variations in the intensity, position and number of precipitin bands. Rocket immunoelectrophoresis of p30 showed the lowest 1/100 dilution to have the same result as the highest dilution at 1/900.

The A and B levels, when determined by AI, were greater than the H levels in the same samples except for one. The range of ABH levels varied from 1/300 to over 1/9000. The relative proportions of A to H and B to H as determined by AI were not always duplicated by AE.

In conclusion, the two qualitative tests, acid phosphatase and p30, have not shown to be appropriate for quantitative determinations. Due to the large variation in ABH levels, it appears to be difficult if not impossible to attribute any detected ABH activity solely to seminal fluid when the victim exhibits the same activity or masks the seminal activity.

THE COMPONENTS OF TOTAL ACID PHOSPHATASE ACTIVITY IN POST-MORTEM VAGINAL SPECIMENS DETERMINED USING INHIBITORS

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In recent years, the determination of quantitative levels of acid phosphatase, although not so much used to identify seminal fluid, is finding growing popularity as a method to determine the amount of seminal fluid present in order to interpret blood typing results obtained from vaginal washings and swabs. Specific questions regarding the meaning of acid phosphatase determinations first surfaced during a statistical review of the results obtained from post-mortem vaginal specimens submitted for seminal fluid identifications. These statistics showed that a large percentage (60 percent) of cases surfaced in which acid phosphatase, electrophoretically identical to the seminal variety (SAP), was found in cases where seminal fluid could not be proven. Saline extracts of selected post-mortem vaginal swabs taken at the time of autopsy were analyzed electrophoretically and quantitatively for acid phosphatase activity in the presence of the inhibitors tartrate and fluoride, specific anti-human serum that reacts with prostatic acid phosphatase, and after heat denaturation. The results have shown that even when seminal fluid can be shown to be present, the total acid phosphatase content may be due to enzyme activity from different sources. The results of representative cases are shown below.

In some cases, essentially all the phosphtase activity was totally inhibited (cases A and I); in others, a

RESULTS OF INHIBITOR STUDIES

	Micromoles p-Nitrophenol Produced								
Inhibitor	*A*	*B*	*C*	*D*	*E*	*F*	*G*	*H*	*I*
None	43	130	130	66	38	130	120	28	70
Tartrate	1	27	14	13	17	110	7	6	1
Fluoride	1	4	1	14	24	42	1	9	1
Heat	1	42	77	12	18	3	98	12	3
Semen present	+	+	+	+	+	+	+	–	–

substantial fraction of the total enzyme activity was due to a heat-stable component (cases B, C, G); and in one case (F), the bulk of the enzyme activity was tartrate-stable although seminal fluid was present. In other examples where an anti-human prostatic acid phosphatase serum was used, the total enzyme activity was comprised of two components: one heat stable and the other due to the presence of enzyme activity removable by the antiserum. These results suggest that interpretations regarding the presence of seminal fluid or the amount present based on total enzyme activity may be misleading.

EFFECTS OF STORAGE CONDITIONS ON THE STABILITY OF PROSTATIC ACID PHOSPHATASE IN SEMEN STAINS

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The qualitative demonstration of prostatic acid phosphatase (PAP) is widely used as a presumptive test for semen stains. It had been suggested that quantitative determinations of the levels of PAP and a semen-specific glycoprotein, prostate antigen (p30), may be useful in predicting levels of soluble ABH blood-group substances in dried semen stains or vaginal swabs. Before such a correlation can be established, however, the stability of PAP in dried semen stains must be examined. Consequently, the present study was designed to quantitate PAP activity in semen stains stored at −20°, 4°, 22° and 37° C under dry and moist conditions. The results demonstrated that the PAP activity recovered from dried semen stains decreased with an increase in storage temperature, while the concentration of protein recovered from these stains remained constant. Semen stains stored under moist conditions lost PAP activity more rapidly than those maintained dry. A parallel study revealed that the use of phosphate-buffered eluants during the extraction or assay of PAP can lead to decreased enzyme activity.

PRESUMPTIVE SCREENING TEST FOR SEMINAL ACID PHOSPHATASE USING SODIUM THYMOLPHTHALEIN MONOPHOSPHATE

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The forensic scientist, when confronted with clothing or material involving sexual assault, requires a rapid, sensitive screening test for seminal fluid. The use of sodium thymolphthalein monophosphate for seminal acid phosphatase testing is examined as an alternative to the substrate α-naphthyl phosphate and o-dianisidine. Recent evidence reveals that the dye (o-dianisidine) used in conjunction with α-naphthyl phosphate is a carcinogenic hazard. Ninety-seven tests were conducted on control, vaginal and saliva swabs and clothing from rape kits to compare both substrates. Results of tests using sodium thymolphthalein and α-naphthyl phosphate correlated exactly. Standard saliva, vaginal and seminal fluid were also examined. A five-minute reaction time was minimal for good color development with moderate acid phosphatase levels, while not allowing low level substances (vaginal fluid) to develop much, if any, color. To date no carcinogenic effects have been reported for sodium thymolphthalein according to the National Institute of Occupational Safety and Health Clearinghouse for Health Information. This substrate serves as an effective alternative for preliminary seminal fluid analysis because of its high degree of selectivity, stability and elimination of a potential hazard.

A MICROCAPILLARY ABSORPTION-INHIBITION METHOD FOR THE DETERMINATION OF LEWIS PHENOTYPES FROM SEMEN AND SALIVA STAINS

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A simple low-cost method for the detection of the Lewis^a (Le^a) and Lewis^b (Le^b) antigens to determine secretor status from body fluid stains other than blood has been developed. Combining a microcapillary hemagglutination method with absorption-inhibition, dried semen stains from 200 individual Caucasians were screened for Lewis phenotypes. Among the semen samples tested, the frequency of Lewis (a – b –), (a + b –) and (a – b +) was 7 percent, 20.5 percent and 72.5 percent respectively, confirming the reliability of this method. Lewis a and b antigen titers were compared in liquid semen samples from 77 individual secretors and 32 individual non-secretors. The titer of the Le^a antigen in non-secretors ranged from 1/32 to 1/4096, while in secretors the titer of the Le^b antigen ranged from 1/16 to 1/4096. Interestingly, the Le^a antigen was detected in semen samples in one-third of the Lewis a –b + individuals with titers ranging from 1/4 to 1/128. In testing repeat semen samples from a single donor, levels of the Le^a and Le^b antigens varied up to three serial dilutions. Studies comparing saliva and semen revealed higher titers for both the Le^a and Le^b antigen in saliva. The Le^a antigen in Le^{a+b-} individuals can be up to six times higher in saliva than in semen. Similarly, the Le^b antigen in Le^{a-b+} individuals can be up to 20 times higher in saliva than in semen.

APPLICATION OF THE CAPILLARY LEWIS INHIBITION TECHNIQUE FOR FORENSIC SAMPLES

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The use of the capillary tube technique for Lewis typing via absorption-inhibition has been modified from Mudd (Presentation at Southern Association Forensic Science, Tallahassee, Florida, September 5 –6, 1980) and Baer & Kearney (Presentation at 35th Annual Meeting American Academy Forensic Science, Cincinnati, Ohio, February 19, 1983) in the typing of population standards or controls and of actual case material (since 1981). The assays were performed using a simple, easily constructed apparatus originally described by Long (presented at 1st Combined CA and N.W. Association Forensic Science, Stateline, Nevada, November 4–6, 1981). Based on this experience, the assay should be performed on cell-free extracts, as opposed to cuttings, be performed on several extract dilutions and with at least two titers of antisera near the endpoint of agglutination. The assay has the advantages of giving exceptionally clear endpoints but requires more manipulation that the microtiter plate assay of Gibbons *et al.* (paper B –15, presented at 35th Annual Meeting American Academy Forensic Science, Cincinnati, Ohio, February 15 –19, 1983) and is therefore less suitable for timed observation or extensive serial dilution studies on individual samples. Experience shows that the technique is most useful in confirming secretor status on poor quality saliva samples and on saliva stains (such as cigarette butts), although it can be used to confirm the lack of secretor substances mixed in semen-vaginal fluid stains as long as the work of Gibbons *et al.* (paper B –16, presented at 35th Annual Meeting American Academy Forensic Science, Cincinnati, Ohio, February 15 –19, 1983) is considered in the interpretation.

CASE REPORT Le^{a-}, Le^{b+} NON-SECRETOR?

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A sexual assault case was received in the laboratory which seemed to be a routine case until it was learned that the assailant was suspected of committing a number of rapes in the Chicago suburbs.

A sexual assault evidence kit and panties from the victim were submitted to the laboratory. The kit and panties were analyzed and seminal material was identified on the panties. Analysis of the whole blood standards indicated that the victim was an ABO type O non-secretor Le^{a+}, Le^{b-} and the suspect was an ABO type O secretor Le^{a-}, Le^{b+}. The saliva sample from the victim confirmed her non-secretor status; however, the saliva sample from the suspect gave conflicting results: no ABO blood-group substances could be detected by the absorption-inhibition method. The saliva standard was tested for the presence of amylase which was positive and absorption-inhibition was repeated giving the same results. Finally, another saliva sample was requested; amylase was present but again no ABH blood-group substances could be detected.

The next logical step was to determine the Lewis antigens that were present in the stains. The victim's saliva sample was typed as Le^{a+}, Le^{b-}, consistent with a non-secretor; the suspect's saliva sample was typed as Le^{a-}, Le^{b+}, consistent with his whole blood standard. The panties were typed in Lewis and both the Le^{a} and Le^{b} antigens were present. On the basis of these results, the suspect could not be eliminated as being a possible source of the seminal material present. Gm typing further confirmed this; the victim was a Gm −1, −2 and the panties were positive for Gm 1 and negative for Gm 2.

The suspect was found guilty of rape and sentenced to 30 years in prison. Although the saliva sample was not quantitatively tested for the amount of ABH blood-group substances present, the only conclusion drawn was that the suspect had very low levels of blood-group substances in his secretions that could not be detected by the absorption-inhibition method currently used.

AN INHIBITION THIN-LAYER IMMUNOASSAY METHOD FOR THE DETECTION OF p30 IN SEMEN STAINS

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The use of p30 as a semen marker has been described previously by Sensabaugh (J. For. Sci., 23: 106, 1978). We have developed a thin-layer immunoassay (TIA) for the detection of this protein in semen stains. Antigen monolayers were prepared in polyvinyl chloride microtiter wells using diluted seminal plasma. A 2 μl aliquot of anti-p30 which had been inhibited with a stain extract was incubated on this monolayer. Following this incubation, anti-rabbit IgG was applied to the monolayer to reinforce the antigen-antibody reaction if it had occurred. The p30 protein was detected in 0.25 μl stains which had been stored at room temperature for approximately 1 year and in semen stains which had been heated at 100 ° C. Blood, saliva, urine, perspiration and tears did not interfere with the method. The detection of p30 by TIA may be a useful component of an analytical protocol for the identification of semen stains since it is sensitive, rapid and requires a small volume of extract. For example, 20 5 μl aliquots of stain extracts may be analyzed in approximately two hours with a detection limit of approximately 50 ng.

THE DISTRIBUTION OF SEMINAL MARKERS ON VARIOUS SECTIONS OF STAINS FROM SEXUAL ASSAULT CASES

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Male ejaculated semen is known to consist of a complicated mixture of cells, fluids, inorganic and organic substances from various glands and sources. These various components of semen are released in a sequential manner and vary in composition. During the course of examining stains from sexual assault cases, we have observed that the stains were not uniform in composition. Various sections of the stain may contain different amounts of seminal markers.

Control semen, seminal stains, vaginal swabs and stains from rape cases were used to examine the distribution of sperm, acid phosphatase, choline, spermine, ABH blood-group substances and PGM1 isoenzymes. The distribution of these markers on various areas of the stain has important implications for sample selection from evidential materials in sexual assault cases which are to be subjected to analysis for identification and genetic markers.

Our studies determined that there are three distinct portions (areas) on semen stains. In the center portion of the stain the cellular debris, including sperm, seems to be concentrated. In the outer portion, or periphery of the stain, the proteins and ABH blood-group antigens seem to be concentrated. The mid-portion of the stain represents an area where seminal components are present but not concentrated as just described.

SIMULTANEOUS ELECTROPHORESIS OF PHOSPHOGLUCOMUTASE (PGM) AND PEPTIDASE A (PEP A)

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PGM and Pep A are the enzymes found in seminal fluid in demonstrable amounts that are routinely typed. Our laboratory during the past year has routinely performed electrophoresis of PGM and Pep A simultaneously with excellent results. It was our observation that approximately 60 percent of our sexual assault cases involved black suspects and Pep A could be used as an identifier of this race. In addition, increasing caseload, the submission of a minimal amount of test samples in rape kits and the growing demand for scientific evidence in the courtroom were some of the reasons for combining PGM and Pep A into one electrophoretic assay. These reasons provided the impetus for abandonment of the conventional multi-enzyme systems Group I and Group IV (Group I—phosphoglucomutase, esterase D and glyoxalase; Group IV—peptidase A, glucose-6-phosphate dehydrogenase and carbonic anhydrase II; personal communication, B. Wraxall, Emeryville, CA) electrophoresis on semen stains.

A modification of the Group I system was made, therefore, for the adaptation and simultaneous electrophoresis of PGM and Pep A. The system uses a 1 percent agarose, Sigma type V, with 1 percent starch gel run at 400 V for 2½ hours. EDTA is deleted from the Group 1 tank buffer (pH 7.4) and 0.1M Clelands is used as the reducing agent. The development of PGM is similar to that described in a conventional Group I run with the addition of EDTA (0.01M) to the PGM reaction buffer. The overlay is poured from the origin to 6 cm anodic of the origin. Pep A is developed as described in the Group IV system with the overlay poured in the area directly anodic to PGM. Incubation is at 37 ° C for approximately one hour. Esterase D can also be developed prior to PGM development as on a conventional Group I plate.

DEVELOPMENT OF A RADIAL GEL DIFFUSION TECHNIQUE FOR THE IDENTIFICATION OF URINE STAINS

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Most of the procedures available to date for urine stain identification are time-consuming, tedious, costly and require a large stain size according to forensic science standards.

A radial gel diffusion method utilizing urease and bromthymol blue has been developed for urine stain identification. Urea present in urine in relatively high concentrations can be detected from urine stain extracts. This technique provides both qualitative and quantitative results and is sensitive enough to detect 0.078 ug/μl of urea.

Two percent agar (w/v) gel is boiled, then cooled to 48 -50 ° C. Bromthymol blue solution and urease are added. Adjust to pH 5.8 -6 with 0.1N H_2SO_4. Pour approximately 3 ml of the liquid agar solution into plastic petri dishes (50 x 9 mm) and allow to solidify. Blank plates are made the same way without adding urease. When the test is run, make 1 mm diameter wells in the gel. Add urine extract (0.5 cm^2 urine stain extracted in 0.2 ml water), 1 μl per well, for 8 to 10 minutes. A dark blue ring will appear if the stain is urine. A standard curve can be made by plotting the concentration of urea in ug/μl vs. the ring diameter in mm^2. The linearity range of urea determined by the standard curve is 0.156 to 0.62 μg/ml.

THE REMOVAL OF SALTS FROM SMALL SAMPLES OF SEMEN

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The high salt concentration of semen, and particularly semen stains, can distort electrophoretic separations of the proteins found in semen. Dialysis can remove these salts and, thereby, eliminate this electrophoretic problem. Unfortunately, dialysis of samples less than 0.5 ml is rather difficult. A technique described by Awdeh (Anal. Biochem. 71: 601, 1976), which spots samples onto a porous cellophane membrane that lies on a buffer-carrying medium (glass wool), allows one to dialyze and retrieve samples as small as 1 μl and as large as 50 μl within several hours. However, the efficacy of this dialysis technique cannot be realized with the commercially available cellophane membranes. Samples applied to cellophane or dialysis tubing spread a great deal which may result in mixing of adjacent samples as well as prevent retrieval of a particular sample. This spreading can be alleviated by boiling a cellophane membrane in a solution containing 0.05 M ammonium bicarbonate and 0.01 M EDTA for two hours. This dialysis technique with the treated membrane can be set up and maintained in any laboratory at a minimal cost and greatly facilitates the dialysis of microsamples of semen, thereby enhancing electrophoretic separation of proteins found in semen.

THE OCCURRENCE OF FOREIGN HAIR AND ITS GENETIC MARKER DETERMINATION IN SEXUAL ASSAULT EVIDENCE

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The Regional Criminalistics Laboratory in Kansas City, Missouri, received 319 sexual assault cases in calendar year 1982. Of the total cases received, 170 cases were examined (55 percent) utilizing an average of 5.9 hours of exam time per case. It becomes apparent that consideration of the efficacy of the analytical

approach to sexual assault trace evidence is important.

Of the 170 cases examined, spermatozoa were identified in 85 (50 percent) of the cases and high levels of acid phosphatase identified in an additional 20 (12 percent) of the cases. A discrete seminal stain ideally suited for genetic marker determination was rarely located. In 22 cases (13 percent), the stain was located in the victim's underclothing. In the remaining cases the identification was made on either a swab or slide prepared from the swab. In contrast, a foreign hair (one which can associate victim and suspect) that is ideally suited for comparison was identified in 53 (31 percent) of the cases. Collection of such hair is normally limited to pubic hair combings, the victim's or suspect's clothing and sweepings from the surface upon which the assault occurred.

Numerous genetic markers can be determined from other than naturally fallen hairs for significant periods of time after hair loss. The suitability of a hair for electrophoretic exam can be determined microscopically prior to permanent mounting. Simultaneous determination of esterase D, phosphoglucomutase and glyoxalase I yields a discrimination power of 0.88 for the Caucasian population. Electrophoresis is carried out with the root of the hair inserted in the gel while the shaft is left laying on the gel surface. After electrophoresis, the hair is mounted in the appropriate medium for microscopic examination.

THE IMPORTANCE OF TESTING SEXUAL ASSAULT VICTIMS FOR VENEREAL DISEASE

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Syphilis serologic and bacteriologic studies are an often overlooked facet of sexual assault evidence. Testing for treponemal antibodies and for gonococci at the time of the victim's initial examination usually detects only pre-existing infections. Retesting after an appropriate interval may identify an infection as being consistent with having been contracted at the time of the assault.

For evidentiary use, however, the test results at both times are important. Venereal disease transmission from assailant to victim or from victim to assailant has the potential for providing dramatic corroboration of the assault and identity of the assailant.

Conversely, while venereal disease is clearly contagious, a single sexual contact with an infected partner does not guarantee contracting venereal disease. Rather, the rate of infection is significantly lower and differs for males and females.

In the Minneapolis–St. Paul, Minnesota area, experience in several thousand sexual assault examinations has shown that a small (but significant) percentage of sexual assault complainants are infected with gonorrhea at the time of initial examination. In contrast, a positive syphilis serologic test is only rarely encountered. As a result, prosecutors and defense attorneys are asking questions about the significance and interpretation of venereal disease testing with increasing frequency.

THE TRUTH CONCERNING THE FEMALE EJACULATE

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Within the past several years, newspaper articles have appeared referencing a publication (Addlego *et al.* J. Sex Research 17:13, 1981) proclaiming the presence of a female ejaculate. This ejaculate is described as containing high levels of prostatic acid phosphatase. The problems for forensic serologists which could arise from these statements are self-evident. If these claims, attributed primarily to one of the coauthors of the referenced publication, Beverly Whipple, Assistant Professor of Nursing, Gloucester County College, Sewell, New Jersey, are true, the finding of prostatic acid phosphatase in sexual assault cases

would no longer be proof positive of semen present, thus confirming intercourse. Therefore, our laboratory requested samples from Whipple and subsequently received samples from three different female ejaculators. No prostatic acid phosphatase was found in any of the samples by two methods, enzyme assay and electrophoresis. One sample contained an acid phosphatase with the same electrophoretic mobility as standard vaginal acid phosphatase. The other two samples contained acid phosphatase with a much greater anodal mobility than prostatic acid phosphatase. In addition, the relative activity of the acid phosphatase in the three samples was much lower than in semen.

The information obtained in our laboratory by testing these samples may be valuable to forensic serologists in refuting claims that prostatic acid phosphatase located on evidence in sexual assault cases could have originated from a female ejaculate.

SECTION III
WORKSHOPS

WORKSHOPS

This section represents the handouts of presentations made by invited scientists who provided demonstrations in three different areas of analysis of sexual assault evidence. As you read each of these three sections you will note that there are several methods described for detecting the same substance. For staining spermatozoa and vaginal epithelial cells a total of 15 different staining methods were presented. For detecting ABO and Lewis blood-group substances there were three different methods. There were different methods for detecting prostate antigen (p30) and prostatic acid phosphatase. Based on the presentations and discussions between the members of the audience and those making the presentations it became apparent that all of the methods presented were reliable. No one method was preferred and each had some aspect that made that specific method desirable.

WORKSHOP I

STAINING AND EXTRACTION TECHNIQUES

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SAMPLING TECHNIQUES

Following is a description of some of the more commonly used procedures that have been used to separate dried seminal stains and seminal materials from their substrata. A few references are included, and some of these are intended to indicate the original proposer of the method.

Scrapings

Once a stain has been located, the crusty area can be scraped from the substratum, usually by means of a scalpel. This procedure can be done with a dried stain, or one that has been wetted with a variety of different solutions (*Hamlin 1883; Williams 1937*).

Teasing

Fibers from the stained area can be "teased" out using sharp pointed forceps or a similar tool and examined. Some authors have recommended this procedure in cases where there are well-defined visible individual fibers in the substratum (*Mezger 1857*).

Cutting

A portion of the stain can be cut out from the overall stain-containing article. The method is especially useful for textile materials.

Tape Lifting

Stains found on surfaces which cannot readily be scraped, cut or collected in another way can be lifted from the substratum. Both sticky tapes and gum acacia have been used for this procedure (*Gabbi 1914; DeBernardi 1959*).

Examine Stain As Is

If a stain were deposited on a small surface, such as the end of a Q-tip, etc., the stained fibers may be examined directly. This procedure has also been suggested for stains on nylon fibers (e.g., stockings).

Destruction of the Substratum

Some authors have recommended chemical destruction of the substratum using sulfuric acid or another chemical which does not obliterate sperm cells (*Green and Burd 1946; Vogel 1882*).

EXTRACTION TECHNIQUES

The following are some extraction/separation techniques that are or have been commonly used and recommended. The objective of these procedures is to separate spermatozoa from substratum materials and, to some extent, from other debris in the stain.

1. Extraction with water
2. Extraction with saline
3. Extraction with detergent solutions
4. Extraction with ammonia solutions (*Bayard 1839*).
5. Extraction in the presence of enzyme solutions (*Bohne and Dieckman 1956*).
6. Extraction—filtration (*Bayard 1839; Ellis 1960*).
7. Extraction—centrifugation (*Corin 1907*).
8. Extraction—Centrifugation in Colloidal Silica Gel (*Takatori and Sasaki 1980*).
9. Extraction—Sonication (*Kivela 1964*).

MICROSCOPICAL IDENTIFICATION OF SPERMATOZOA

1. Examination without histological staining
   (a) Light microscope
   (b) Phase-contrast microscope
2. Examination with crystal test reagents
   (a) Florence reagent
   (b) Barberio reagent
3. Examination of histologically stained preparations (Details presented in Section IV.)

HISTOLOGICAL STAINING AND STAINING TECHNIQUES

Many different histological staining methods and procedures have been described for seminal fluid smears, spermatozoa, seminal fluid-vaginal secretion mixtures and other components of vaginal smears.

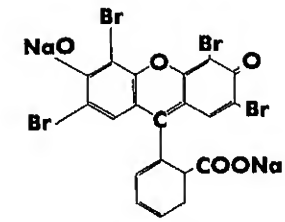
The following is a list of histological stains and stain combinations that have been used for these purposes. Following the list, there is a detailed description of the stains, their preparation, procedures for employing them and a brief description of the appearance of spermatozoa, seminal fluid and vaginal smears that have been stained using a particular method.

1. Wright's Stain
2. Harris Hematoxylin-Eosin
3. Harris Hematoxylin-Eosin-Indigocarmine
4. Acid Fuchsin-Methylene Blue
5. Basic Fuchsin
6. Gentian Violet-Rose Bengal
7. Crystal Violet-Tannic Acid
8. Carbol Crystal Violet
9. Gentian Violet-Carmine
10. Ammoniacal Erythrosin
11. May-Grunwald or Jenner's Stain
12. Giemsa Stain
13. Nuclear Fast Red-Picroindigocarmine Stain ("Christmas Tree")
14. Papanicoleau Stain
15. Stains-All
16. Bryan-Leischmann Stain

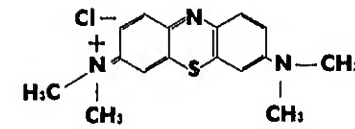
1. Wright's Stain

A. *General description*

This stain was designed principally for blood smears, especially for differentiating white blood cells. It consists of Eosin Y (CI 45380) containing oxidized, partially demethylated Methylene Blue (CI 52015). It can be purchased commercially and commercial preparations work well.



Eosin Y



Methylene Blue

B. *Preparation of Stain*

(1) 0.3 g Wright's stain in 100 ml absolute methanol (acid free); allow to stand 1 to 2 days

(2) Lillie's modification: 1 g Wright's stain in 50 ml glycerol mixed with 50 ml methanol is the stock solution. Just prior to use, 4 ml stock solution is mixed with 3 ml acetone, 2 ml 67 mM phosphate buffer, pH 6.5, and 31 ml distilled water.

C. *Staining Technique*

1. Prepare smear or film on slide.
2. Fix smear 2 min by standing in methanol and allow to dry.
3. Add sufficient staining solution to completely cover the specimen and allow to stand for 2 min.
4. Add 67 mM phosphate buffer, pH 6.5, to the staining preparation in about twice the quantity of the original stain solution added. This effectively dilutes the stain *in situ*. Let stand 5 minutes.
5. Carefully flood off the stain with 67 mM phosphate buffer, pH 6.5, to wash the specimen and allow to dry.

D. *Appearance of Stained Preparations*

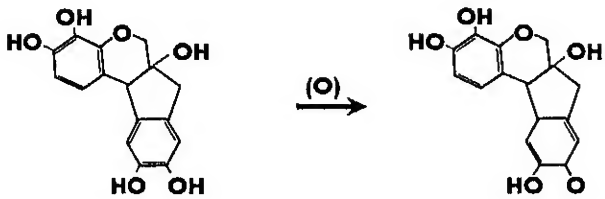
Sperm:	Epithelial Cells:
head → purple	nucleus → darker purple
midpiece → unstained	cytoplasm → light purple
tail → dark	(contrast not too good)

(*Wright 1902; Conn 1940; Lillie 1965*).

2. Harris Hematoxylin-Eosin Y

A. *General Description*

This stain has been used primarily for differentiation of nuclei. It consists of a Hematoxylin (CI 75290) stain and an Eosin Y (CI 45380) counterstain. Hematoxylin has been prepared a number of different ways over the years for different purposes and the solutions tend to carry the name of the original proposer. Hematoxylin itself is not a dye, but its oxidation product, hematein, which results from the preparation of the dye solution, is a biological dye.



Hematoxylin **Hematein**

B. *Stain Preparation*

(1) Harris Hematoxylin—5 g Hematoxylin; 2.5 g HgO (red); 50 ml absolute ethanol; 100 g ammonium or potassium alum; 1 liter distilled water.

Dissolve the hematoxylin in alcohol, and the alum in the water with heating. Remove from heat and mix the two solutions. Bring to a boil as quickly as possible (in less than 1 minute). Remove from heat, slowly add the HgO and reheat to a simmer until a dark purple color results. Remove from heat and plunge the vessel into cold water to cool. Two to 4 ml glacial acetic acid should be added per 100 ml stain solution just prior to use. It is important to know whether commercially obtained Harris hematoxylin has the acetic acid added or not; some procedures call for the use of this preparation without the acetic acid.

(2) Eosin Y—1 g water soluble Eosin Y is dissolved in 20 ml distilled water. Eighty ml 95 percent ethanol is added. This is stock eosin solution. Working solution consists of 1 part stock solution plus 3 parts 80 percent alcohol; 0.5 ml glacial acetic acid is added just prior to use.

C. *Staining Technique*

1. Fix slide containing smear quickly and gently in a flame.
2. Stain in Harris Hematoxylin for 15 minutes.
3. Rinse stain off gently with tap water.
4. Quickly dip 3 to 10 times in acid alcohol (1 liter 70 percent alcohol containing 10 ml concentrated HCl).
5. Check preparation with microscope to ensure that nuclei are distinct.
6. Wash very briefly with tap water.
7. Dip 3 to 5 times in ammonia water (1 liter tap water containing 2 to 3 ml 28 percent ammonia) or saturated $LiCO_3$ (1 g $LiCO_3$ in 100 ml distilled water) until specimen is bright blue.
8. Wash gently with running tap water 10 to 20 min.
9. Stain with Eosin Y solution 30 sec.
10. Fix 2 min in 95 percent ethanol.

D. *Appearance of Stained Preparations*

Sperm: anterior head → unstained
posterior head → purple
midpiece → blue-purple
tail → dark to light yellow

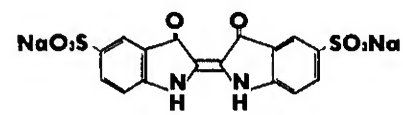
Epithelial Cells:
nucleus → dark blue-purple
cytoplasm → light red-purple

(*Luna 1968; Nickolls 1956; Rentoul and Smith 1973*).

3. Harris Hematoxylin-Eosin Y-Indigocarmine

A. *General Description*

This stain was designed for vaginal smears. It consists essentially of a Hematoxylin (CI 75290) stain step with Eosin Y (CI 45380) and Indigocarmine (CI 73015) counterstains.



Indigocarmine

B. *Stain Preparation*

(1) Harris Hematoxylin; see Stain #2.

(2) Solution B: 70 ml absolute ethanol, 28.8 ml distilled water and 1.2 ml concentrated ammonia.

(3) 0.25 g Indigocarmine and 0.75 g Eosin Y in 99 ml distilled water; add 5 drops $CHCl_3$.

C. *Staining Technique*

1. Prepare smear; allow to dry completely.
2. Immerse in 95 percent ethanol for 3 min.
3. Transfer slide to distilled water for 1 min.
4. Transfer to Harris Hematoxylin for 20 min.
5. Wash in running water for 2 min.
6. Immerse in Solution B (above) for a few seconds.
7. Transfer to Indigocarmine-Eosin solution overnight.
8. Rinse quickly in tap water.

9. Dip 5 times in each of 20 percent, 90 percent and absolute ethanol and allow to dry.

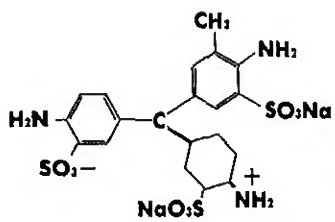
D. *Appearance of Stained Preparations*

Sperm: anterior head → light blue to unstained
posterior head → purple
midpiece → purple-blue
tail → dark to light yellow
Epithelial Cells:
nucleus → dark purple-blue
cytoplasm → light blue
(*Cuyler 1932, Gurr 1965*).

4. Acid Fuchsin-Methylene Blue

A. *General Description*

This is a stain made from Acid Fuchsin (CI 42685) with a Methylene Blue counterstain (CI 52015).



Acid Fuchsin

B. *Preparation of Stain*

(1) 0.2 g Acid Fuchsin in 100 ml distilled water.
(2) 1 g Methylene Blue and 1 g K_2CO_3 in 100 ml distilled water; ripen 1 to 2 weeks; dilute with distilled water (1:10) for use (Polychrome Methylene Blue).

C. *Staining Technique*

1. Use dried smear; fix for 2 min in methanol.
2. Allow to dry.
3. Stain in Acid Fuchsin for 8 min.
4. Wash with distilled water for 2 min.
5. Stain in methylene blue for 1 min.
6. Wash in distilled water by dipping for 1 min.
7. Immerse in absolute ethanol for 15 sec and allow to dry.

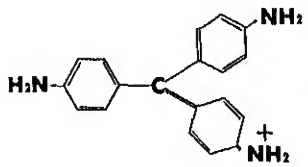
D. *Appearance of Stained Preparation*

Sperm: anterior head → blue
posterior head → purple-blue
midpiece → blue
tail → lightly blue-stained
Epithelial Cells:
nucleus → purple-blue
cytoplasm → light blue
(*Baecchi 1909; Strassmann 1921*).

5. Basic Fuchsin

A. *General Description*

Basic fuchsin is primarily Pararosanilin (CI 42500) and is a component of the Feulgen stain used to demonstrate chromatin in plant and animal cells.



Basic Fuchsin

B. *Stain Preparation*

25 mg basic fuchsin in 10 ml distilled water

C. *Staining Technique*

1. Use dried smears.
2. Stain in basic fuchsin 2 min.
3. Wash with distilled water by flooding gently for 1 min.
4. Allow to dry.

D. *Appearance of Stained Preparation*

Sperm: anterior head → pink
posterior head → red
midpiece → pink
tail → dark
Epithelial Cells:
nucleus → red
cytoplasm → pink
(*Laudermilk 1941; Baima-Bollogne 1968*).

6. Gentian Violet-Rose Bengal

A. *General Description*

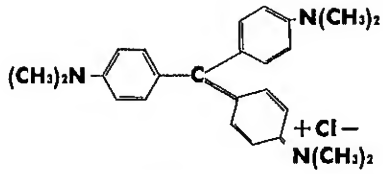
"Gentian Violet" is a well-known stain that is really a poorly defined mixture of violet rosanilins. Today, purer compounds are employed to make up this stain: either Crystal Violet (CI 42555) or Methyl Violet 2B (CI 42535). The former tends to give a deep violet color, the latter a more reddish shade. Rose Bengal, the counterstain, is Rose Bengal Tr (CI 45440).

B. *Preparation of the Stain*

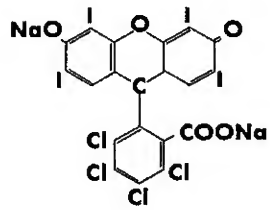
(1) 0.5 g Crystal Violet in 100 ml distilled water (Methyl Violet 2B can be substituted for Crystal Violet)
(2) 1 g Rose Bengal in 100 ml distilled water

C. *Staining Technique*

1. Use a smear that is dry; fix gently with flame.



Crystal Violet



Rose Bengal

2. Immerse in 95 percent ethanol 1 min.; allow to dry.
3. Stain in Crystal Violet solution 3 min.
4. Wash by dipping 5 times in water, 10 times in 95 percent ethanol and again 5 times in water.
5. Stain in rose bengal for 1 min.
6. Wash in water until background is cleared up of stain particles.
7. Allow to dry.

D. *Appearance of Stained Preparation*

Sperm: anterior head → pink-purple
posterior head → purple
midpiece → pink-purple
tail → dark
Epithelial cells
nucleus → pink-purple
cytoplasm → pink-light purple
(*Holbert 1936*).

7. Crystal Violet-Tannic Acid

A. *General Description*

This is a Crystal Violet (CI 42555) staining technique which involves immersion of the specimen in tannic acid as well as in the dye solution. The structure of Crystal Violet is shown in Stain #6.

B. *Preparation of Stain*

(1) 5 g tannic acid in 100 ml distilled water
(2) 0.5 g Crystal Violet in 100 ml distilled water

C. *Staining Techniques*

1. Use a smear that has been air dried.
2. Briefly fix with flame.
3. Immerse in 5 percent tannic acid solution for 30 min.
4. Wash by flooding gently with water.
5. Immerse in Crystal Violet solution for 3 min.
6. Wash by flooding gently with water.
7. Allow to dry.

D. *Appearance of Stained Preparations*

Sperm: anterior head → light blue
posterior head → dark blue
midpiece → light blue
tail → dark
Epithelial Cells:
nucleus → poor contrast
cytoplasm → purple
Staining is not particularly good; there is some apparent distortion of sperm cell morphology; contrast is poor. (*Macaggi 1925; Clark 1973*).

8. Carbol Crystal Violet

A. *General Description*

This stain is a Crystal Violet (CI 42555) made up in phenol. The structure of the dye is shown in #6.

B. *Preparation of the Stain*

1 g Crystal Violet in 100 ml 2 percent aqueous phenol and 30 ml absolute ethanol

C. *Staining Technique*

1. Use air dried smear.
2. Fix briefly with flame.
3. Immerse in stain solution 5 min.
4. Wash by flooding gently with distilled water 1 min. or until the background is clear.
5. Allow to dry.

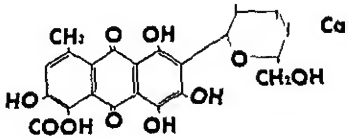
D. *Appearance of Stained Preparation*

Sperm: anterior head → blue-purple
posterior head → purple
midpiece → purple
tail → dark
Epithelial Cells:
nucleus → purple
cytoplasm → light purple
(*Gurr 1965; Paulsen and Varnek 1953*).

9. Gentian Violet-Carmine

A. *General Description*

See the comments on Gentian Violet in #6 Crystal Violet or Methyl Violet 2B may be used. Carmine is carminic acid (Tr) (CI 75470) and is one of the oldest histological stains. Carmine stains glycogen a reddish color, which may be helpful in examining the vaginal epithelium.



Carminic Acid

B. *Preparation of Stain*

(1) 0.5 g Crystal Violet (CI 42555) in 100 ml distilled water; (Methyl Violet 2B, CI 42535) may be substituted.

(2) Carmine: Boil 2 g Carmine with 1 g K_2CO_3 and 5g KCl in 60 ml distilled water until color darkens. Cool and add 20 ml 28 percent ammonia water. Let sit 24 hrs. Store cold. This is the stock solution. For use: 8 ml stock solution is added to 12 ml 28 percent ammonia water and 24 ml methanol. The solution should be used immediately; this dilute solution does not keep.

C. *Staining Technique*

1. Use air-dried smear. Fix gently with flame.
2. Stain in Crystal Violet solution for 3 min.
3. Rinse with water by dipping 5 times.
4. Stain 20 min. in the diluted Carmine solution.
5. Rinse by dipping 5 times each in water, 80 percent ethanol, then 95 percent ethanol and allow to dry.

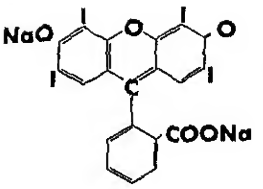
D. *Appearance of Stained Preparations*

Sperm: anterior head → light purple
posterior head → pink
midpiece → light purple
tail → dark
Epithelial Cells:
nucleus → pale purple
cytoplasm → light purple
(*Weigert 1887; Best 1906*).

10. Ammoniacal Erythrosin

A. *General Description*

Erythrosin B (CI 45430) is the tetraiodo compound corresponding to the tetrabromo compound that is typical Eosin. It is not very stable in ammonia solution.



Erythrosin B

B. *Preparation of Stain*

(1) 1 g Erythrosin B in 100 ml methanol

(2) Bring 2 g $K_2Cr_2O_7$, 1 g Na_2SO_4 and 25 ml ammonia to a final volume of 100 ml with distilled water

(3) Dilute 1 part Erythrosin solution with 1 part ammonia solution for use.

C. *Staining Technique*

1. Use dried smear.
2. Fix briefly with flame.
3. Stain for 8 min in working solution.
4. Wash by flooding gently with water.
5. Air dry.

D. *Appearance of Stained Preparations*

Sperm: anterior head → light pink
posterior head → dark pink
midpiece → pink
tail → dark
Epithelial cells:
nucleus → dark pink
cytoplasm → light pink
(*Corin and Stockis 1908; Tsunenari* et al *1971*).

11. May-Grünwald or Jenner's Stain

A. *General Description*

These stains were designed primarily for blood smears and act similarly to Wright's. Many authors consider May-Grünwald and Jenner's stains as identical; others say that Jenner's has more Eosin Y. These are essentially Methylene Blue-Eosin Y stains and can be obtained commercially.

B. *Preparation of Stain*

0.25 g May-Grünwald stain powder in 100 ml absolute methanol

C. *Staining Technique*

1. Use a dried smear.
2. Immerse in stain 3 min.
3. Rinse off by flooding with water and allow to dry

D. *Appearance of Stained Preparations*

Sperm: anterior head → light blue
posterior head → dark blue
midpiece → light blue
tail → light blue
Epithelial Cells:
nucleus → dark blue
cytoplasm → light blue
(*May and Grünwald 1902; Jenner 1899; Mueller 1926*).

12. Giemsa Stain

A. *General Description*

Giemsa stain is complex and has a long and complicated history. Currently, Giemsa in the United States is made from Azure A Eosinate, Azure B Eosinate, Methylene Blue Eosinate and Methylene Blue Chloride in glycerol and methanol. Giemsa stain is normally obtained commercially The stain was developed for blood smears, particularly for detection of malarial parasites in blood.

B. *Preparation of Stain*

(1) 0.9 g Giemsa in 33 ml glycerol; allow to stand 1.5 to 2 hours at 60 °. Add 33 ml methanol and let stand one day. This is a stock solution. For use; Bring 1.5 ml stock to 30 ml final volume with distilled water; or

(2) 0.8 g Giemsa in 100 ml of an equal mixture of glycerol and methanol; shake or stir mechanically for 2 to 3 days. This is a stock solution. For use: 1 volume stock in 50 volumes 0.01M phosphate buffer, pH 6.5.

C. *Staining Technique*

1. Use an air dried smear.
2. Immerse in methanol 5 min.
3. Allow to air dry.
4. Stain 50 min in working Giemsa solution (time can be varied from 15 to 50 min depending upon stain preparation, age, etc.).
5. Wash by gently flooding with distilled water.
6. Allow to dry.

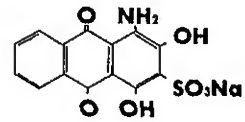
D. *Appearance of Stained Preparations*

Sperm: head → blue-green to green
midpiece → blue-green to green
tail → dark
Epithelial Cells: greenish to unstained; contrast poor between nucleus and cytoplasm.
(*Giemsa 1902; Raitzin 1928*).

13. Nuclear Fast Red-Picroindigocarmine Stain

A. *General Description*

This stain was developed for spermatozoa and for seminal vaginal mixtures. It consists of a Nuclear Fast Red (CI 60760) stain followed by an Indigocarmine (CI 73015) in picric acid counterstain. Nuclear Fast Red is also called Calcium Red; its German name is Kernechtrot. This stain is sometimes called "Christmas Tree" because the spermatozoa are stained different colors in different parts. Heads are reddish, midpieces blue and tails a yellowish green. These colors also contrast well with the green to blue-green shades of the epithelial cells.



Nuclear Fast Red

B. *Preparation of Stain*

(1) Nuclear Fast Red (may be called "Kernechtrot Solution" or "KS"): 5 g $Al_2(SO_4)_3$ in 100 ml hot distilled water; add 0.1 g Nuclear Fast Red. Stir, allow to cool and filter.

(2) Picroindigocarmine (PICS): Add 4 g picric acid to 300 ml water, cover and allow to stand, giving a saturated solution. One g Indigocarmine is dissolved in this solution and the resulting solution filtered. A few picric acid crystals may remain.

C. *Staining Technique*

1. Use air dried smear. Fixation is unnecessary.
2. Cover sample area with KS solution and place in moisture chamber for 15 min (may be placed under inverted Petri dish).
3. Wash with water by gentle flooding.
4. Cover sample area with PICS solution for 15 sec or less.
5. Wash and fix by gently flooding the slide with absolute ethanol.
6. Allow to dry.

D. *Appearance of Stained Preparations*

Sperm: anterior head → light red
posterior head → dark red
midpiece → blue
tail → yellowish green
Epithelial Cells:
nucleus → green-blue green
cytoplasm → light green-blue green
(*Oppitz 1969*).

14. Papanicoleau Stain

A. *General Description*

Papanicoleau (or "Pap") stain is a complicated stain which has undergone many modifications over the years. It was originally designed for studies of vaginal smears in exfoliative cytology. Pap staining involves Harris Hematoxylin staining, acidification, Orange G staining and a complex counterstain called "EA 50" (it was called "EA 36" in the original paper). EA 50 has Light Green SF, Bismark Brown and Eosin Y dyes.

B. *Preparation of Stain*

(1) Harris hematoxylin (see #2)
(2) 0.03N HCl
(3) 0.5 g Orange G (CI 16230) and 0.015 g phosphotungstic acid in 100 ml 95 percent ethanol
(4) EA 50 Counterstain: 4.5 ml of a 0.5 percent alcoholic solution of Light Green SF Yellowish (CI 42095), 10 ml of a 0.5 percent alcoholic solution of Bismark Brown Y (CI 21000), 45 ml of a 0.5 percent alcoholic solution of Eosin Y (CI 45380), 200 mg phosphotungstic acid and 1 drop saturated $LiCO_3$. (Some of these solutions are available commercially and as part of staining kits.)

C. *Staining Technique*

1. Use air dried smear.
2. Fix in 1:1 ether-absolute ethanol solution
3. Immerse in Harris hematoxylin (without acetic acid in it) for 5 min.
4. Wash with 6 dips in distilled water.
5. Dip 8 times in the HCl solution (2) above.
6. Wash in running tap water 6 min, then dip 30 sec in distilled water and 30 sec in 95 percent ethanol.
7. Immerse in Orange G solution (3) above for 90 sec.
8. Dip slowly twice in 95 percent ethanol.
9. Immerse in EA 50 solution (4) above for 90 sec.
10. Dip slowly twice in 95 percent ethanol and then for 30 sec in absolute ethanol.
11. Allow to dry.

D. *Appearance of Stained Preparations*

Sperm: anterior head → blue
posterior head → purple-blue
midpiece → purple
tail → dark
Epithelial Cells:
nucleus → purple-blue
cytoplasm → very light blue
(*Papanicoleau 1941; Lillie 1965*).

15. Stains-All Stain

A. *General Description*

This stain is made from 1-ethyl-2-[3-(1-ethylnaphtho [1,2d]thiazolin-2-ylidene)-2-methylpropenyl]-naphtho [1,2d]thiazolium bromide. Solutions of the dye have been found to stain DNA, RNA, protein and mucopolysaccharides different colors and the dye solution has been called "stains-all."

B. *Preparation of the Stain*

Stock solution is 0.1 g dye dissolved in 100 ml formamide. Working solution is prepared by mixing isopropanol (50 ml) with 10 ml stock solution, 10 ml formamide, 1 ml 3M Tris HCl, pH 8.8, and distilled water to a final volume of 200 ml. Staining solution is light sensitive; store in the dark.

C. *Staining Procedure*

1. Use air dried smear.
2. Stain by immersion in working solution 30 min in the dark.
3. Rinse with water by gentle flooding.

D. *Appearance of Stained Preparation*

Sperm: anterior head → clear to light blue-green
posterior head → blue green
midpiece and tail → unstained
Epithelial Cells: poorly differentiated.
(*Dahlberg* et al *1969; Green* et al *1973*).

16. Bryan-Leischmann Stain

A. *General Description*

This stain is complex and has undergone a number of modifications over the years. It was designed for studying sperm and seminal fluid morphology in smears.

B. *Preparation of Stain*

(1) Modified Bryan's Stain: 0.5 g Eosin Y (CI 45380), 0.5 g Fast Green FCF (CI 42053), 0.5 g Naphthol Yellow S (CI 10316) dissolved in 1500 ml 1 percent acetic acid; filter before use.
(2) Leischmann Stain: 0.5 g eosinated Methylene Blue dissolved in 300 ml absolute methanol; mix thoroughly. Allow to age in the dark at room temperature for 7 days. Incubate at 35°C for 2 days. Working solution: 50 ml above stock solution in 150 ml phosphate buffer, pH 6.8 (Wright's or Jenner's stains may be used as alternatives).
(3) Pyronin Y: 0.1 g Pyronin Y (CI 45005) is dissolved in 4 ml aniline and 96 ml 40 percent ethanol.

C. *Staining Technique*

1. Use air dried smear.
2. Fix in 10 percent alcoholic formalin (1 min), then in 80 percent ethanol, 70 percent ethanol and 50 percent ethanol (5 min each).
3. Immerse in α-naphthol solution briefly (1 g α-naphthol in 100 ml 40 percent ethanol and 0.2 ml 3 percent H_2O_2).
4. Rinse in tap water by gently flooding.
5. Immerse in Pyronin Y solution 4 min.
6. Rinse in tap water by gently flooding.
7. Immerse in sodium citrate buffer, pH 6.8, 3 min.
8. Wash in distilled water by gently flooding.
9. Immerse in modified Bryan stain 15 min.
10. Wash by gently flooding with 1 percent acetic acid.
11. Rinse by gently flooding with tap water.
12. Immerse in Leischmann stain 30 min.
13. Rinse briefly in distilled water by gently flooding.
14. Allow to dry.

D. *Appearance of Stained Preparations:*

Sperm: posterior head → deep red
anterior head → unstained to pink
midpiece → deep red
tail → pale yellow
(*Belsey* et al *1980*)

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WORKSHOP II

PROSTATE ANTIGEN/P30 AND PROSTATIC ACID PHOSPHATASE

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The write-up on prostate antigen (p30) representing the presentation on methods by Dr. Baechtel appears in the chapter prepared by Dr. Baechtel titled Immunological Methods for Seminal Fluid Identification on page 83.

THE EFFECT OF ENZYME SUBSTRATE ON ACID PHOSPHATASE DETERMINATIONS

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The highly elevated levels of prostatic acid phosphatase present in human semen have elicited a great deal of attention from forensic scientists. In most forensic laboratories, the qualitative demonstration of prostatic acid phosphatase is still used as a presumptive test for suspected seminal stains, thereby providing a rapid means for screening a large number of stains. In the FBI Laboratory, it was estimated that this figure could reach as high as 4000 individual assays per month.

In 1971, Roy *et al.* introduced sodium thymolphthalein monophosphate (STMP) as a substrate for prostatic acid phosphatase. Their work demonstrated that STMP, when compared to other commonly used substrates, was the substrate least likely to react with a non-prostatic source of acid phosphatase (AP). Consequently, STMP has been adopted by many forensic laboratories, including the FBI Laboratory, as the substrate of choice for the presumptive AP assay.

In selecting a substrate for use in a presumptive assay, it is important to realize that the activity of an enzyme, in units/ml, associated with a particular tissue or body fluid will vary dramatically with the type of substrate employed. This variation in enzyme activity is a reflection of the specificity of the enzyme for a given substrate.

This effect is demonstrated in Table 1 which shows the change, quantitatively, in the acid phosphatase activity obtained from different sources when assayed with different substrates.

Table 1. THE EFFECTS OF ENZYME SUBSTRATES ON THE ACID PHOSPHATASE ACTIVITY FROM VARIOUS SOURCES

AP Source	*DPP*	*pNPP*	*αNP*	*PP*	*STMP*
Semen	1590[b]	1820	550	87.0	620
Vaginal[a]	0 072	0.066	0.022	0 0058	0.020
Serum	0.55	0.0074	0.0018	0.0029	N.A.[c]
Saliva	0.042	0 027	0.0092	0 0050	0.0090
Erythrocyte[a]	0.52	0.26	0.0060	0.11	0.0056
Liver[a]	0 21	0 11	0.0040	0.045	0 010
E Coli[a]	0.14	0.0069	0.0022	0.0027	N.A.[c]

[a]Enzyme activity determined from extracts.
[b]Units/ml.
[c]No activity observed.

Those substrates tested included disodium phenylphosphate (DPP), p-nitrophenylphosphate (pNPP), alpha-naphthylphosphate (α-NP), phenylphthalein phosphate (PP) and sodium thymolphthalein monophosphate (STMP).

For each source of acid phosphatase studied, an equal concentration of sample was assayed with the five substrates and the results given in units/ml (units = micromoles of product per minute). As shown in Table 1, large variations in the hydrolysis rates were observed for the substrates when assayed with the different AP sources.

Because an enzyme such as AP may have different rates of hydrolysis for different substrates, caution must be taken in comparing the levels of AP (in units/

ml) when determined by two different methods In some cases the differences in activity determined for a specific specimen may be due to a difference in the substrate used in the assay rather than a quantitative difference in the amount of enzyme present.

If the different assay conditions used to determine the AP levels were known, it should be possible to determine empirically a conversion factor which would permit the comparison of these different AP levels.

By using the seminal AP levels determined with pNPP and STMP from Table 1 as an example, a conversion factor for the transformation of STMP units into pNPP units can be calculated by dividing the units of pNPP by STMP (pNPP/STMP = 2.9).
In calculating a conversion factor for two substrates, it should be recognized that a separate conversion factor must be determined for each AP source, since differences in substrate specificity may exist. Further, the substrate concentration for the assays in question, as well as assay temperature and pH, must be the same as in those assays used to calculate the conversion factor.

When establishing a presumptive assay for prostatic (seminal) AP, the assay conditions (substrate concentration, pH, incubation time and sample size) should be set in such a way that only the high AP levels associated with seminal fluid would produce a positive color change.

Inasmuch as the levels of AP in semen can vary from individual to individual, the absence of a positive color change does not preclude the presence of semen since, in some specimens, the levels of AP may be below the minimum level necessary to produce a positive result. This effect is shown in Table 2 using the presumptive STMP assay. From these results, it was determined that those semen specimens having more than 50 units/ml would produce a positive color change. From the results of a distribution study of AP in over 200 semen specimens, it was found 2 percent of the specimens had AP levels below that necessary to produce a positive result.

As previously discussed, the selection of a substrate is very important in establishing a presumptive assay for AP. Keeping in mind the relative rates of substrate hydrolysis for the different acid phosphatases, a substrate that was least reactive with non-prostatic sources of AP would therefore be less likely to produce a positive color change when these acid phosphatases were encountered in routine casework. In addition to the substrate, other assay parameters such as substrate concentration, incubation temperature and enzyme concentration will also affect the results of a presumptive assay. These factors should also be considered when comparing the results of two enzyme assays.

Table 2. RELATIONSHIP BETWEEN PROSTATIC ACID PHOSPHATASE LEVELS IN NEAT SEMEN AND THE PRESUMPTIVE STMP ASSAY

Sample Number	*Acid Phosphatase pNPP Units/ml*	*Presumptive STMP*
WF-6	14.2	—[a]
WF-5	21.7	—
WF-18	42.6	w[b]
WF-41	51.5	+[c]
WF-39	86.6	+
WF-1	159	+
WF-21	393	+
WF-29	646	+
WF-52	1027	+
WF-136	1414	+

[a]Negative color change.
[b]Weak color change.
[c]Positive color change.

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ACID PHOSPHATASE ASSAY OF VAGINAL SWABS

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Method

p-nitrophenyl phosphatase quantitative procedure.

Principle

Acid phosphatase (ACP) catalyzes the cleavage of phosphate from p-nitrophenyl phosphate producing p-nitrophenol (pNP) which has a yellow color in alkaline solution; the extent of the reaction is measured by the change in absorbance at 410 nm.

Specimen Preparation

Swab of vaginal vault; when saturated, typical swabs contain about 0.1 ml vaginal vault fluids.

1. Cut swab tip in half. One half is used for the ACP assay and the remaining half is preserved for other analyses, e.g., genetic marker typing.

2. Place half of swab into test tube and extract with 0.2 ml Tris buffered saline (TBS) at 0–5°C for one-half to 1 hour.

3. Remove swab material and express liquid against side of tube using a surgical-gloved finger; about 75 percent (v/v) of the intitial 0.2 ml aliquot should be recovered. (The amount recovered does not affect calculations, but it does affect subsequent testing if antigen typing is necessary.)

4. Centrifuge extract at 1200–1500 rpm.

5. Transfer supernatant to a new test tube; this is the cell-free extract used for the acid phosphatase assay.

6. Suspend the cellular debris in distilled water and centrifuge, discarding the supernatant wash. Aspirate the cellular material with bulb syringe and place on microscope slide; fix by drying in an oven at 55°C for ½ hour; stain slide with Christmas tree stain/sperm stain.

Assay Procedure

1. With a portion of the neat extract (from step 5 above), prepare a 1/10 dilution with TBS; keep cold.

2. Prepare 4 labeled tubes containing assay buffer as follows:

   a. Control—1.5 ml assay buffer
   b. Sample (neat)—0.5 ml assay buffer
   c. Sample (1/10)—0.5 ml assay buffer
   d. Blank—0.5 ml assay buffer

3. To tubes #1 and #2, add 10 µl extract; to tube #3, add 10 µl of the 1/10 dilution.

4. Add 1 ml substrate solution to tubes #2 to 4; time reaction from this point.

5. Stop reaction at 20 minutes by addition of 1.75 ml sodium hydroxide to each tube.

6. Read absorbance of each in spectrophotometer (1 cm cuvette) at 410 nm using water to "zero" instrument.

Interpretation and Calculations

1. A blank reading higher than 0.050 indicates significant non-specific hydrolysis of substrate and the substrate solution should be checked.

2. The control should read lower than the blank. Higher readings suggest the presence of blood; the Soret absorption of hemoglobin is 406 to 430 nm.

3. The amount of ACP present in the cell-free extract is calculated as follows:

The amount of pNp (in µmoles) produced in an assay tube is:

$$\frac{(A_{410}\text{ sample} - A_{410}\text{ blank}) \times 3.25\text{ ml}}{16200\text{ L/mole cm}} = 0.2(A_{410}\text{ sample} - A_{410}\text{ blank})$$

where 16200 L/mole cm is the molar absorption coefficient of pNP in alkaline solution at 410 nm. One international unit (IU) of acid phosphatase activity is defined as the amount of enzyme which will turn over one µmole substrate per minute. The conditions of the assay (0.01 ml sample assayed, 20 minutes assay time) have been adjusted so that the ACP activity of the sample in IU/ml is

$$\frac{0.2\,(A_{410}\text{ sample} - A_{410}\text{ blank})\ \mu\text{moles}}{.01 \times 20} = (A_{410}\text{ sample} - A_{410}\text{ blank})$$

Example 1: If the (A_{410} sample – A_{410} blank) for the neat extract is 0.890, then the extract contains 0.89 IU/ml. The 1:10 dilution of that extract should have an (A_{410} sample – A_{410} blank) of about 0.089, i.e., 0.089 IU/ml.

4. The assay is linear to a (A_{410} sample – A_{410} blank) of about 1.5; should the neat sample give values exceeding this, the 1:10 dilution reading should be used. If it also exceeds a 1.5 reading, further dilutions should be made and assayed.

Example 2: The (A_{410} sample – A_{410} blank) for the neat extract exceeds 2.0 and the value for the 1:10 dilution is 0.56 IU/ml. The 1:10 dilution thus contains 0.56 IU/ml and the neat extract contains 5.6 IU/ml.

5. The assays may be done at 37° C instead of room temperature. Assay values at 37° C will be 2.2 times higher than assay values at 20° C.

Discussion and Clinical Significance

The determination of ACP activity levels in the vaginal fluids of alleged rape victims can yield evidence of the presence of semen and an estimate of how much semen is present. The latter estimate aids in the interpretation in genetic typing tests (e.g., ABO typing) run on the vaginal material and may provide information about postcoital intervals.

1. Evidence of semen is provided if the determined ACP level is significantly higher than the endogenous vaginal ACP level. The endogenous vaginal ACP activity recovered from dried swabs using the above procedure will average about 0.06 IU/ml. Sensabaugh (1979) indicates that 99 percent of endogenous ACP levels fall below 0.4 IU/ml and 99.9 percent fall below 0.85 IU/ml. Values higher than these may be considered significantly elevated, i.e., indicative of the presence of semen. The effects of vaginal infection and pregnancy on these values are not known.

2. Estimates of the effective semen dilution in a swab extract and the amount of semen present on the swab are based on the distribution of ACP levels in semen

(Blake *et al.* 1981). Using the described assay, 90 percent of semen samples have ACP levels below 600 IU/ml. Thus a high estimate of the semen dilution factor in a swab extract can be made by dividing the number of IU in the extract into 600; the true dilution factor will be lower 90 percent of the time.

Example 1: $\frac{600 \text{ IU/ml}}{0.89 \text{ IU/ml}} = 674$

i.e., a 1:674 dilution

Example 2: $\frac{600 \text{ IU/ml}}{5.6 \text{ IU/ml}} = 107$

i.e., a 1:107 dilution

The low estimate of the amount of semen present (in μl) on the swab is calculated in similar fashion:

$$\frac{(\text{Extract IU/ml, } (0.2 \text{ ml}) (1000 \ \mu\text{l/ml})}{(0.5 \text{ swab}) (600 \text{ IU/ml})} = \frac{(0.67 \text{ ml } \mu\text{l}) (\text{Extract IU/ml})}{\text{IU swab}}$$

The actual amount will be higher 90 percent of the time.

Example 1: (0.67)(0.89) = 0.60 μl/swab

Example 2: (0.67)(5.6) = 3.8 μl/swab

3. The above calculations can be used as a guide to subsequent genetic testing (Blake *et al.* 1981). Semen dilutions 1:100 or less should yield meaningful ABO typing. Swabs containing 5 μl or more semen usually can be typed for phosphoglucomutase (PGM) (Blake and Sensabaugh 1978).

4. Postcoital intervals cannot be estimated with any precision (Sensabaugh 1979). However, swab ACP values exceeding certain threshold values place limits on postcoital intervals. For example, swab ACP values exceeding 25.7 IU/ml are unlikely beyond 6 hours postcoitus; swab values exceeding 4.4 IU/ml are unlikely beyond 12 hours postcoitus.

5. Swab ACP values below the significance threshold do not counterindicate sexual activity. Fifteen percent of swabs collected 3 to 6 hours postcoitus exhibit non-elevated ACP levels and the proportion increases with time (Sensabaugh 1979).

6 Swabs are best preserved by drying, freezing or both. Leaving them moist at room temperature for any extended period should be avoided; these conditions are conducive to sample deterioration.

Methods and Reagents

1. Test tubes (10 × 100)
2. Assay buffer:
   0.1 M acetate, pH 5.5
   Acetic acid—5.75 ml
   H_2O—make to 1 liter
   Adjust to pH 5.5 with 10 M NaOH.
3. Assay substrate:
   3 mM p-nitrophenyl phosphate—28 mg
   Assay buffer—25 ml
   (Prepare fresh daily.)
4. Extraction buffer:
   Tris buffered saline, pH 7.4
   Tris base—1.21 g
   NaCl—85.0 g
   H_2O—make to 1 liter
   (Adjust to pH 7.4 with HCL.)
5. Spectrophotometer at 410 nm
6. Pipettes (10 ml)
7. Pipettes (2 ml)
8. Assay stop solution: 1 N NaOH
   Sodium hydroxide—20 g
   H_2O—make to 500 ml
9. Clinical centrifuge
10. Cotton swabs

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WORKSHOP III

INHIBITION METHODS FOR ABH AND LEWIS TYPING

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PARALLEL INHIBITION AND ELUTION TECHNIQUES ON SECRETION STAIN EXTRACTS

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Extraction Procedure

1. Select stain material (including appropriate standards and controls) and transfer to 10 × 75 mm glass test tube.

Material	*Size*	*Distilled Water*
Fabrics	(0.5 × 0.5) cm – 1 cm^2	450 µl
Facial tissue	1 cm^2 (4 ply)	450 µl
Swabs	Entire	450 µl*
Cigarette butts	0.5 × 2.5 cm shell	450 µl
Average-size stamps	Entire	550 µl

*Or 250 µl/swab for pooled swab pairs.

2. Saliva stains: passive extraction at 52° C for 3 hours. Other stains: passive extraction at room temperature for 20 minutes, followed by 10 seconds active, rotary agitation (MOTO-TOOL).

3. Squeeze excess extract from substratum with applicator stick and place substratum in a perforated LP2 tube (perforated at the bottom with a hot needle). Transfer the extract to a non-perforated LP2 tube.

4. Place the perforated LP2 tube on top of the non-perforated LP2 tube and put both inside a 10×75 mm glass test tube.

5. Centrifuge at 2,000 g for 10 minutes.

6. Collect maximum possible supernatant for ABH assay (and Le^a, Le^b assay, if required).

7. If the pellet is required for microscopy, add one drop of distilled water and make a smear.

Dilution Procedure

1. Arrange six 10×75 mm glass test tubes so that the following dilutions can be made: 1/5, 1/10, 1/50, 1/100, 1/200. The first tube will receive the neat extract (supernatant).

2. Using distilled water as a diluent, make the dilutions as follows:

Dilution	*µdistilled water as diluent*	*Dilution sequence by µl transfer**
Neat	0	
1/5	320	+ 80 from neat
1/10	200	+ 200 from 1/5
1/50	400	+ 100 from 1/10
1/100	200	+ 200 from 1/50
1/200	200	+ 200 from 1/100

*A FIN pipette (200 µl) can be used for this task.

Inhibition and Elution Procedures

Materials for Inhibition Grouping

Metal racks.

Nine plastic LP2 tubes for each sample extract arranged in 3×3 pattern. These tubes are set up so that inhibition grouping can be performed on the neat, 1/5 and 1/10 extracts only.

Materials for Elution Grouping

Flat polycarbonate sheets ruled with black magic marker in order to accommodate two samples at 5 dilutions each versus the 3 antisera (i.e., a 3×5 pattern for each sample) or the well-slide polycarbonate sheet ruled with black magic marker to section off eight

sample grouping areas of 15 wells each in a pattern of 3×5 (i.e., each area of 15 wells is to be used for one sample).

Dispensing Sample Extract

1. With the plastic tubes for inhibition and the polycarbonate sheets for elution appropriately labelled in a corresponding pattern, dispense at the rate of 20 µl using the Eppendorf Repeater fitted with a Combitip (cut-off)/disposable tip combination (Combitip, 500 µl; disposable tip, 100 µl).

2. For absorption-elution dispense from dilutions: 1/200, 1/100, 1/50, 1/10 and neat. For absorption-inhibition dispense from dilutions: 1/10, 1/5 and neat.

Absorption-Inhibition Procedures

1. Centrifuge the samples at approximately 1000 g for 5 to 10 seconds to allow the samples to be firmly placed at the bottom of each tube.

2. Make up the appropriate dilutions for each antiserum in 5.5 percent bovine albumin (polymerized).

3. Using the Eppendorf Repeater (fitted with disposable tip) dispense at the rate of 20 µl from each antiserum in designated areas along the length of each rack.

4. Centrifuge the racks at approximately 1000 g for 5 to 10 seconds to ensure that all of the dispensed antisera are put in contact with the samples.

5. Mix.

6. Cover the tubes with lengths of scotch tape and allow to stand overnight at 4° C.

7. Using the Eppendorf Repeater (fitted with disposable tip) dispense 20 µl of 1 percent suspensions of A2, B and O indicator cells to corresponding levels along the length of the racks. Mix.

8. Allow to stand at room temperature for one hour.

9. Centrifuge the racks at 700 g for one minute.

10. Read for agglutination after dislodging the buttons very gently.

Score macroscopically positive agglutination according to the Ortho Diagnostics scale: 4, 3^2, 3, 2^2, 2, 1^2.

Any tube which is read macroscopically as negative is to be read microscopically using an inverted microscope.

Microscopically-positive agglutination is read using the remainder of the Ortho Diagnostics scale: 1, 0^2, 0.

Absorption-Elution Procedure

1. After the samples have been placed on the polycarbonate sheets using the Eppendorf Repeater, the plates are dried on the bench at room temperature overnight. The plates may also be dried at 52° C for one hour.

2. Make up suitable dilutions of the antisera in 3 percent bovine serum albumin (BSA) (polymerization not required).

3. Using pasteur pipettes, add one drop of antiserum to the dried residues at corresponding levels.

4. Allow absorption to take place for at least three hours at 4° C in moisture chambers.

5. Dip each plate once in a large beaker of 4° C saline and immediately submerge the plate into a large saline basin and allow to wash passively for 1½ hours at 4° C.

6. Prepare 0.05 percent cell suspensions of group A1, group B and group O in approximately 0.3 percent BSA (polymerized).

7. Remove one plate from the saline bath at a time. Shake off excess saline with a quick flick of the wrist, and immediately place the plate face down onto a layer of absorbent tissue paper (3 to 4 layers of Kleenex tissues will be sufficient). Quickly repeat the drying step on a fresh layer of Kleenex. Immediately turn the plate face up. Allow for complete drying to take place before proceeding.

8. Place each of the dried plates in a preheated moisture chamber. With a pasteur pipette, add one drop of the prepared cell suspensions of appropriate group to the center of each delineated area where the dried residues are present.

9. Carefully place the moisture chambers in the incubator at 52° C and incubate for 20 minutes.

10. Carefully remove the moisture chambers from the incubator and gently ease out the plates and place them in moisture chambers at room temperature.

11. Rotate for 60 minutes at 100 rpm.

12. Read microscopically on a scale from 4 + to -.

Inhibition Technique for Le^a and Le^b on Secretion Stain Extracts

Materials for Inhibition Grouping

Metal racks (as for ABH grouping technique).

Six plastic LP2 tubes for each sample extract arranged in a 3 × 2 pattern. These tubes are set up so that inhibition grouping can be performed on the neat, 1/5 and 1/10 extracts only or other dilution sequence if so desired (e.g., neat, 1/10 and 1/20).

Absorption-Inhibition Procedure

1. Using the Eppendorf Repeater set at 20 µl (fitted with disposable tip), dispense once from each dilution for each antiserum.

2. Centrifuge these aliquots at approximately 1000

placed at the bottom of each tube.

3. Make up the appropriate dilutions for each antiserum in 3 percent bovine albumin (polymerized).

4. Using the Eppendorf Repeater (fitted with disposable tip), dispense at the rate of 20 μl from each antiserum in designated areas along the length of each rack.

5. Centrifuge at approximately 1000 g for 5 to 10 seconds to ensure that all of the dispensed antisera are put in contact with the samples.

6. Mix and cover the tubes with lengths of scotch tape and allow to stand overnight at 4° C.

7. Using the Eppendorf Repeater (fitted with disposable tip), dispense at the rate of 20 μl of 1 percent O, Le(a+b−) and O, Le(a−b+) indicator cells at corresponding levels along the length of the racks.

8. Mix.

9. Allow to stand at room temperature for ½ hour.

10. Centrifuge at 400 g for 1 minute.

11. Very carefully draw up the contents of each tube with a pasteur pipette and gently place on a microscope glass slide.

Read by the following scale:

C (complete)—Observed macroscopically
4 +—Observed microscopically
3 +—Observed microscopically
2 +—Observed microscopically
1 +—Observed microscopically
W—Observed microscopically
O—No agglutination

DETECTION OF SECRETED ABH AND LEWIS SUBSTANCES: APPLICATIONS OF PHYSICAL EVIDENCE OF SEXUAL ASSAULT

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Data Sources

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Portions of this document dealing with the determination of the levels of ABH and Lewis blood-group thesis (in preparation), Mary M. Gibbons, University of California, Berkeley.

Introduction

The ABO and Lewis typing procedures which follow are absorption-inhibition methods. They are intended to detect secreted blood-group substances. These methods have been applied to the examination of physical evidence of sexual assault as a part of a larger analytical protocol which has been termed "The Systematic Approach to the Analysis of Semen Evidence."

The linchpin of the Systematic Approach is its use of the quantitative acid phosphatase test or quantitative p30 test to derive an estimate of the minimum amount of semen in a sample. Knowledge of the range of levels of blood-group substances in semen and vaginal fluid can be used to help interpret the significance of the typing results obtained by these methods.

Sample Preparation—General Considerations

Samples to be used in the ABO and Lewis absorption-inhibition studies must be cell-free preparations to ensure the detection of secreted blood-group substances rather than cell-bound antigens. For this reason, the removal of cellular debris from an extract of a stain or swab is an important consideration.

The ABO and Lewis techniques described on the following pages each require 50 microliters (μl) of sample to permit a full titering of the antigens. The following procedure will describe the preparation of a cell-free extract of a swab or stain. The initial extract volume is 200 μl. It is essential to recover as much of the initial extract volume as possible; this is best achieved by centrifugation. To some extent the procedure will require modification to suit the particular equipment available in the user's laboratory; however, it is hoped that it will serve as a useful starting point.

Preparation of Cell-Free Extracts

1. Place half of a swab or 1 square centimeter of stain in a plastic tube and add 200 μl of buffer (isotris, pH 7.4). Allow to soak for approximately 30 minutes, agitating occasionally with a toothpick. Keep on ice during this period or refrigerate.

2. Transfer the swab to a disposable plastic pipet tip and insert the tip into the extraction tube piggy-back style. With the sample in this arrangement, centrifuge briefly at high speed (30 seconds).

3. Using a double drawn pipet, transfer the supernatant to a glass test tube, taking care not to disturb the pellet of cellular debris at the bottom of the extrac-

tion tube You should recover virtually all of the initial 200 µl volume. If the swab or fabric is still wet, centrifuge a bit longer to recover more fluid.

The extract is now ready for quantitative assays such as acid phosphatase or p30. Amylase assays can also be conducted on the extract. Do these prior to inhibition work.

Recovery of the Cellular Debris

Some of the cellular debris will have collected as a pellet in the bottom of the extraction tube. Retain this. Additional debris may be trapped in the swab or fabric matrix.

1. To recover trapped debris from swab or fabric matrix, use a toothpick or probe to transfer the swab or fabric to a separate plastic tube. Extract in approximately 1 ml of water. Agitate briefly with a toothpick.
2. Remove the swab or fabric by twining it around a toothpick, pressing the material against the side of the tube to express the fluid as you do so. Discard the swab or fabric.
3. Use a few drops of water from this tube to resuspend the pellet of cellular debris in the extraction tube and combine the samples.
4. Centrifuge. Withdraw the supernatant and discard it. Resuspend the cellular debris in a few drops of water and transfer to a clean microscope slide for fixing, staining and microscopic examination.

Final Preparation of Extract for Absorption-Inhibition

Following the quantitative enzyme and protein assays that may be desired, 100 to 150 µl of extract fluid should remain in the glass tube. A boiling step is required to prepare the sample for the absorption-inhibition tests. Boiling serves two purposes: It will result in the permanent dissociation of antigen-isoantibody complexes which may exist due to the presence of vaginal fluid or blood: and it will also precipitate excess protein which in turn will reduce the tendency of the indicator cells to undergo roleaux reactions.

1. Place the glass tube containing the cell-free extract in boiling water for 3 to 5 minutes.
2. Centrifuge the sample to remove precipitated protein. Recover the supernatant for inhibition typing. Recover as much fluid as possible.

The samples are now ready for ABO and Lewis typing by absorption-inhibition.

ABO Typing of Water-Soluble Antigens by Absorption-Inhibition (according to the procedure of Blake)

Preparation of Glass Slides

A hydrophobic surface is placed on glass slides 5×7. cm microscope slides or 10×10 cm disposabl thin-layer plate glass) by incubating the glass plates i a 1 percent solution of Prosil or Siliclad. The plates ar washed with water, dried and stored for use.

Preparation of the Antisera

The antisera preparation to be used in the antige assay is selected by preparing serial dilutions of antise rum and lectin in isotris (0.14 M NaCl, 10 mM Tris pH 7.4) containing 1 percent BSA. The antisera dilu tions are tested in a standard three-part system con taining one part antiserum, one part isotris and on part cell suspension; 10 µl aliquots are used. Th antiserum and blank are rotated in a humid chambe for 30 minutes prior to the addition of the cell suspen sion. After the cell suspension is added, the agglutina tion reaction is monitored microscopically (100X) i 10-minute intervals for 30 to 40 minutes. The las antiserum dilution which yields a +3 to +4 agglutinatio reaction after 30 minutes of rotation is selected for th antigen assay. The diluted antisera preparations should be prepared fresh daily. Once a particular batch of antisera has been titered, it should remain constant within one serial dilution over a dilution of 1/200 to 1/800. Lectin preparations are usually at a dilution of 1/20 to 1/80. There is some evidence that the H lectin requires a metal ion for maximum activity; therefore ACD and EDTA solutions should be avoided when using H lectin.

Cell Suspension

0.1 percent cell suspensions are prepared in isotris containing 1 percent BSA and 0.80 M glucose. Fresh cells are preferred. Stock preparations of cells are stable in the isotris-glucose solution for about 5 days. The 0.1 percent cell suspensions should be prepared fresh daily.

Procedure

The sample is added to the antisera solutions and rotated in a humid chamber for at least 20 minutes prior to the addition of the cell suspension. The antigen-antibody reaction is instantaneous provided that there is good mixing of the solution. The agglutination is monitored in 10 minute intervals against a

blank which is included on every plate. A gentle tapping of the edge of the plate after each observation period is recommended. It is frequently found that concentrated protein solutions which lack antigen activity cause the test cells to agglutinate more rapidly than the control blank. In most instances, this phenomenon is merely a protein effect which reduces the natural negative charge on the cells. If the presence of antibody in the sample is suspected, this can be tested in a simple two-part assay. A signal that the phenomenon is caused by antibody would be the observation that the effect is taking place in the A and/or B cells, but not in the O cells. An enhancement of agglutination in the O cells as well as in the A and B cells signals a protein effect. For those samples which contain antigen activity, the titer of the activity should be determined. This will aid in assessing whether that activity is most likely from the victim or from the semen.

Interpretation

There are three possible outcomes of ABH typing of vaginal fluid/semen mixtures.

(1) Antigen is detected which is foreign to the female.

(2) Antigen is detected which is not foreign to the female.

(3) No ABH antigen is detected.

In the first situation, the implications regarding the semen donor are relatively straightforward. To assess the significance of the second and third outcomes, two pieces of information are necessary. They are: (1) knowledge of the range of ABH levels in semen and vaginal fluid as determined by the typing method used in the examination of evidence specimens, and (2) knowledge of the minimum amount of semen in the sample under consideration.

Table 1 summarizes, in a general way, the range of secreted ABH levels in neat semen and neat vaginal fluid. Donors of all ABO types have been combined for this purpose.

Table 1. THE RANGE OF SECRETED ABH LEVELS IN NEAT SEMEN AND NEAT VAGINAL FLUID

	ABH levels (reciprocal titers)
Neat semen (n = 87)	400 - 200,000
Neat vaginal fluid (n = 91)	0 - 4,000

Now, consider the situation where no foreign antigen is detected. One could conclude that the semen was contributing at least some of that antigen if the antigen level in the specimen exceeds the highest levels seen in vaginal fluid. The ABH contribution potential of vaginal fluid can be considerable. Vaginal fluid/semen mixtures having levels of ABH activity exceeding that which could be contributed by vaginal fluid alone will be encountered infrequently.

The significance of the absence of ABH antigen is dependent upon the amount of semen in the sample under consideration. The absence of secreted ABH substances would indicate that the semen donor is a non-secretor if the amount of semen in the sample is sufficiently concentrated to permit detection of ABH substances from the lowest-level secretors (i.e., titers of 1/400).

If, on the other hand, the semen is diluted to such an extent that ABH substances from some secretors would not be detectable, then the absence of ABH activity has no significance. In this case, no information about the ABO type or secretor status of the semen donor would be provided.

Lewis Typing of Secretions by Absorption-Inhibition Using Ficin-Treated Indicator Cells

This procedure is used in the detection and quantitation of soluble Lewis blood-group substances in secretions. It is essential that the samples assayed in this manner be cell-free preparations.

Preparation of Indicator Cells

Freshly drawn or commercially available O, Le(a+) and O, Le(b+) red cells are washed twice in isotris buffer. To approximately 100 μl of packed cells is added 1 ml of 0.05 percent ficin prepared as a solution in isotris containing 0.08 M glucose. Cell suspensions are incubated in a 37° C oven for 15 to 20 minutes. Cells are then centrifuged; the supernatant is withdrawn and discarded. Cells are washed three times in isotris to remove the residual ficin.

Preparation of Cell Suspensions

The cell suspensions used are 0.1 percent suspensions prepared in isotris containing glucose stock. Ficin-treated cells can be used for no more than two consecutive days. The 0.1 percent suspensions must be prepared daily.

Preparation of Glass Slides

A hydrophobic surface is prepared on glass slides by submerging the glass in a 1 percent aqueous solution of Prosil or Siliclad. The plates are washed with water, dried and stored for use. Five by 7.6 cm. microscope slides can be used. Ten by 10 cm plates prepared from 10 × 20 cm thin-layer glass plates are very convenient for running many samples at once.

Selection of the Antisera Dilutions

The anti-Le(a) and anti-Le(b) antisera preparations to be used in the antigen assay are selected by preparing serial dilutions of antisera in isotris. The antisera dilutions are tested in a standard three-part system containing one part antiserum, one part isotris and one part cell suspension; 7 μl volumes are used. The antisera are rotated in a humid chamber for 20 minutes prior to the addition of indicator cells.

After the cell suspensions are added, the agglutination is monitored microscopically (100X) at 10-minute intervals for 30 to 40 minutes. The last antisera dilutions which yield +3 to +4 reactions after 30 minutes of rotation are selected as the working dilutions of antisera to be used in the examination of unknown secretion samples. A gentle tap at the edge of the plate to resuspend the cells is recommended after each reading.

Once the antisera preparations have been titered, the working dilutions should not vary more than one serial dilution from day to day. Different lots of antisera, however, will vary considerably in their agglutinating abilities. Antisera from Ortho Diagnostics have demonstrated working dilution titers ranging from 1/4 to 1/64. It is essential to redetermine the working titer of antisera each day they are used.

Inhibition Procedure

Serial dilutions of a sample are prepared using isotris. Equal volumes (7 μl) of samples and antisera are added side by side on the glass plate. They are mixed by gently tapping the edge of the plate. The plate is placed in a humid chamber and rotated for 20 minutes prior to the addition of the indicator cells. The antigen antibody reaction is quite rapid provided there is good mixing of the samples.

An equal volume (7μl) of indicator cells is added to each sample and the plate is rotated again in the humid chamber. Agglutination reactions are monitored at 10-minute intervals and scored against a blank included on each plate consisting of equal parts antiserum buffer and test cells. A gentle tapping of the edge of the plate at the end of each observation period is recommended.

Inhibition is demonstrated by the complete absence of agglutination in a test sample compared to a fully agglutinated blank after 30 minutes rotation time. The titer is defined as the last dilution of a sample showing complete or virtually complete inhibition. Figure 1

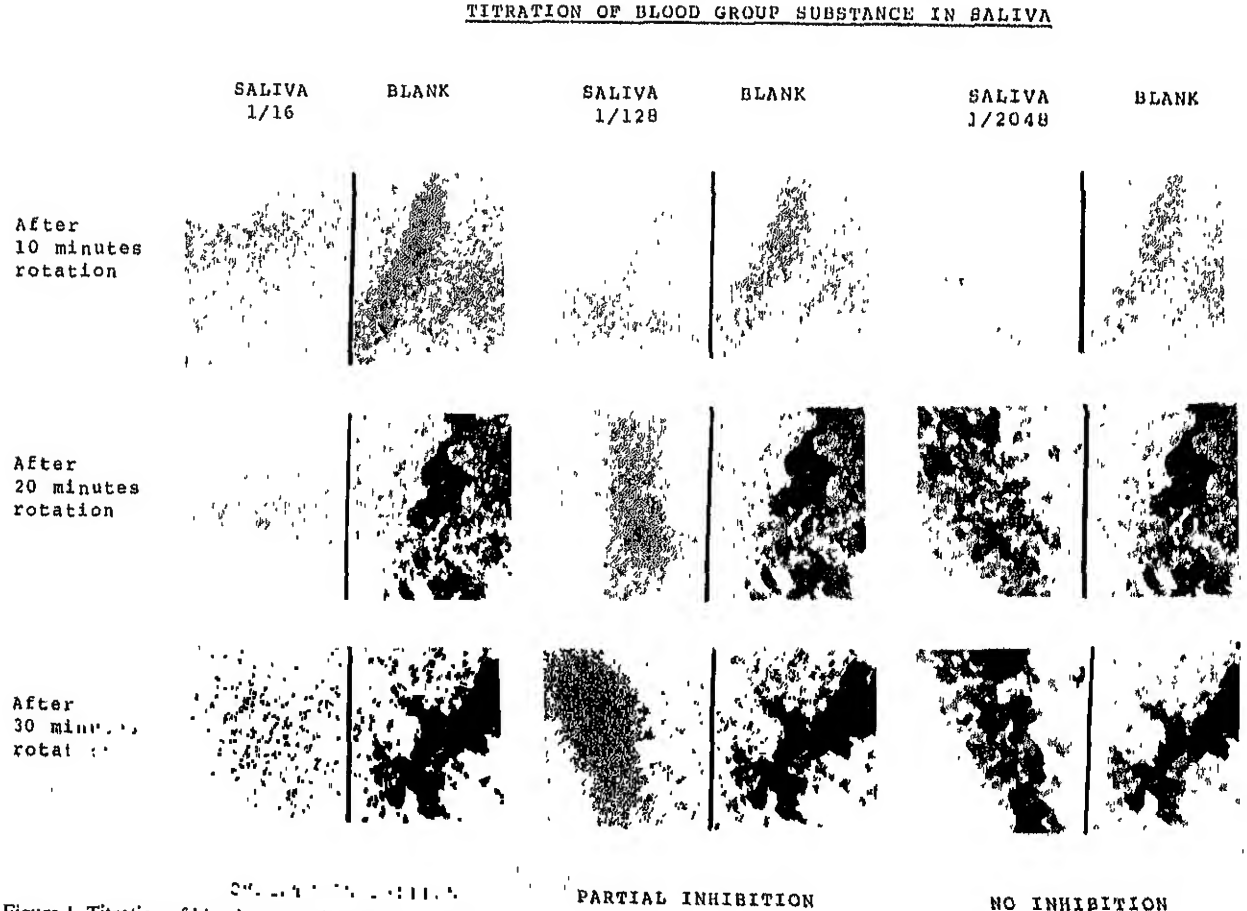

Figure 1. Titration of blood group substance in saliva.

illustrates inhibition and its absence using three dilutions of a saliva sample.

Interpretation

The classical pattern of expression of the Lewis blood-group substances in the secretions and their expression as detected by the microscopic method are summarized in Table 2.

Table 2. THE DETECTION OF LEWIS BLOOD GROUP SUBSTANCES IN SECRETIONS BY THE CLASSICAL AND MICROSCOPIC METHODS

Lewis blood type	*Secretor status*	*Lewis Substances in the Secretions*	
		Classical	*Microscopic*
a+	Nonsecretor	a	$a \gg b$
b+	Secretor	$a \leqslant b$	$a \leqslant b$
a–b–	Secretor	None	Low levels $a \cong b$
a–b–	Nonsecretor	None	a only

Unexpected Lewis activity has been repeatedly detected in Le(a+) and Le(a–b–) individuals. The unexpected Lewis activity appears to be a component of the secretion rather than nonspecificity associated with the antisera. It is unclear at this tme whether the activity is due to genuine Lewis a and b blood-group substances or to some background glycoprotein species common to all individuals and sufficiently similar in structure to allow recognition by the Lewis a and b antibodies. This microscopic technique is 10 to 100 times more sensitive than the classical tube techniques. The detection of these unexpected substances is undoubtedly a result of the increased sensitivity of the method.

Determination of the Lewis blood type from an examination of secretions by this method may at times be ambiguous. The determination will depend not only on the type of Lewis substances detected, but also on the relative amounts. High levels of Le(a) and Le(b) would indicate that the fluid donor is Le(b+). If low levels of both substances are found, it would be unclear whether the donor is a Le(b+) or a Le(a–b–) secretor. If high levels of Le(a) and low levels of Le(b) are detected, a Le(a+) donor would be indicated. In the few samples from Le(a–b–) nonsecretors examined to date, we have found Le(a) substance only; no Le(b) has been detected. It is unclear at this time whether the absence of Le(b) is a diagnostic characteristic of Le(a–b–) nonsecretors. Examination of a larger population of Le(a–b–) nonsecretors will be necessary to answer this question.

For unmixed secretion stains, we have found that the secretor status of the donor can be deduced in most instances by an examination of the Le a/b ratio for the specimen. This observation applies regardless of the Lewis blood type of the donor. The Le a/b ratio is calculated by dividing the titer of Le(a) by the titer of Le(b) for a given specimen. If only one of the two Lewis antigens is detected, the undetected antigen is assigned a value of 1 to avoid division by zero. If no Lewis substances are detected, the ratio is defined as zero.

The ranges of the Le a/b ratios for secretors and nonsecretors are summarized for vaginal specimens and saliva in Table 3.

Table 3. LEWIS a/b RATIOS AND SECRETOR STATUS

	Secretion	*(n)*	*Le a/b*
Secretors	Vaginal	(79)	0019 8
	Saliva	(6)	.06 4
Nonsecretors	Vaginal	(27)	4 -512
	Saliva	(5)	64 1000

It can be seen from this data that secretors and nonsecretors exhibit little overlap in their respective a/b ratio profiles. A few samples from secretors exhibited ratios greater than 1. However, more than 95 percent of secretor samples had Le a/b ratios less than or equal to 1, and most of these were well below 1. Preliminary studies on semen indicate that the same Le a/b ratio patterns apply for secretors and nonsecretors.

In general, an evaluation of the a/b ratio would allow one to determine the secretor status of the donor when the sample is not mixed with fluid from a second individual. For example, in a concentrated extract of an uncontaminated semen stain, the absence of ABH antigens would suggest that the semen donor was a nonsecretor. The determination of a Lewis a/b ratio for that sample fitting the pattern for nonsecretors would independently confirm the nonsecretor status of the semen donor.

The vast majority of semen stains encountered in forensic situations represent mixtures of semen and vaginal fluid. In these cases the contribution potential of vaginal fluid to the Lewis antigen pool must be considered. Our studies indicate that the levels of Lewis substances in vaginal secretions can be very high. Reciprocal titers of 2000 for both antigens, while not common, have been encountered. Nevertheless, when confronted with a vaginal sample containing concentrated semen and exhibiting no ABH activity or very low levels of ABH antigen not foreign to the female, the same approach can be applied to assess whether the semen donor is a nonsecretor.

If the female is a secretor (either Le b+ or a–b–), the determination of a Le a/b ratio which does not fit

the pattern for a secretor would indicate that the semen was contributing preferentially to the Le(a) pool and would further indicate that the semen donor is a nonsecretor. Preliminary studies of semen from nonsecretors indicate that their Le(a) levels are not great and frequently will be inadequate to produce the required shift in the Le a/b ratio from a secretor pattern to one of a nonsecretor. The approach stands its greatest chance of providing an informative outcome when the female is a secretor who happens to produce little or no detectable Le(b).

The Le a contribution potential of females who are Le(a −) nonsecretors is considerable. It is unlikely that the Le(a) levels in a vaginal specimen containing concentrated semen from a nonsecretor would exceed the contribution potential of vaginal fluid alone. Again, this is based upon preliminary evidence that nonsecretor semen does not contain particularly high levels of Le(a) substances. With this combination of individuals (i.e., nonsecretor female and possible nonsecretor semen donor) chances are remote that Lewis typing will provide an informative outcome with regard to the confirmation of the semen donor as a nonsecretor.

In conclusion, the Lewis detection method described has been applied primarily toward the resolution of the secretor status of the semen donor when no ABH substances are detected in concentrated semen samples admixed with vaginal fluid. The chances for resolving the secretor status question are greatest when the female is a secretor who produces little or no Le(b). Lewis blood type of the donor of an uncontaminated secretion often can be determined using this method. However, that determination does not depend solely on the types of Lewis substances detected, but rather upon both the types and relative amounts of those substances.

MICROTITER PLATE ABSORPTION-INHIBITION TEST FOR THE DETECTION OF SOLUBLE BLOOD GROUP SUBSTANCES IN SEMEN AND SALIVA STAINS

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Introduction

This technique is suitable for detecting soluble blood-group substances (BGS) secreted into body fluids such as semen and saliva. The method may be utilized qualitatively to detect the presence of BGS in a specimen extract or to semi-quantify the level of BGS in fluids or extracts. The salient features of this technique are that the entire test from sample extraction to assessment of results requires less than three hours and the end results can be evaluated macroscopically.

Principle

In principle, this technique is the familiar absorption-inhibition procedure adapted to a 96-well microplate. So that truly soluble BGS are detected, the stain is extracted with buffered saline. Aliquots of the extract are combined and incubated with iso-antibody or lectin. Subsequently, indicator erythrocytes are added to test for the presence of uncomplexed antibody or lectin. After centrifugation of the microplate, all erythrocytes are located in a small pellet at the bottom of the plate well. The plate is tilted at an acute angle for about 30 minutes. If the erythrocytes are agglutinated, they will remain as a distinct pellet in the well bottom or slide intact to the edge. If agglutination has been inhibited by soluble BGS, the erythrocytes will run in a smooth stream toward the side of the well.

Reagents

1. HEPES-buffered saline (HBS): 0.144 M NaCl (8.42 g/L); 0.01 M HEPES (2.38 g/L) titrated to pH 7.2 with NaOH.

2. Tween-albumin-HBS (THBS): 1 percent bovine serum (1 mg/100 ml); 1 percent Tween-20 (1 ml/100 ml); q.s. HBS.

3. Anti-A and Anti-B antisera: Ortho Diagnostics. The proper dilutions must be determined empirically. The diluent must be THBS. H lectin: Serological Research Institute (SERI). The dilution must be empirically determined and the diluent must be THBS.

4. Indicator erythrocytes: Ortho Diagnostics, Affirmagen®— groups A and B; Selectogen®- group O. RBC preparation is described in the appendix.

5. V-bottom microplates: Dynatech - #010102602; *HEPES: N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid.

Procedures (Figure 1)

Extraction of specimens

1. Specimen cuttings of 1 cm^2 are placed in the 1.5 ml polypropylene Eppendorf tubes.

2. 250 μl of HBS is added to each cutting. The extraction should take place for at least one hour at room temperature with occasional agitation. Do not vortex. Alternatively the extraction can be accom-

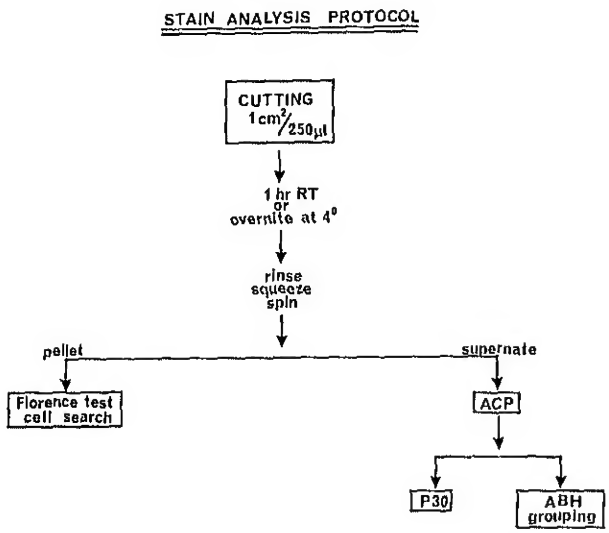

Figure 1 Stain analysis protocol

plished overnight at 4° C. If a smaller cutting has been taken, reduce the volume of HBS proportionally.

3. At the conclusion of the extraction period, the cutting should be pulled out of the extraction fluid and squeezed until most fluid is removed.

4. To maximize the recovery of extraction fluid from the cutting, as well as pellet any cells which might be present, a centrifugation is performed. Punch a small hole (1 mm diameter) in the bottom of a 0.5 mm conical plastic centrifuge tube. Place the squeezed cutting into this tube and insert into the 1.5 ml tube which contains the extract. Centrifuge for one minute.

5. The supernatant may be used to assay soluble semen components such as acid phosphatase, BGS, p30, and Lewis antigens. The pellet (which will be small) may be pipetted directly onto a microscope slide for the Florence test and the search for spermatozoa.

Cell Search

1. Pipette 10 µl from the bottom of the centrifuged stain extract and transfer to a microscope slide.

2. Add 10 µl of Florence reagent and put on a small cover slip.

3. Evaluate for presence of choline crystals and spermatozoa.

Acid phosphatase assay

This test utilizes the acid phosphatose reagent set manufactured by Worthington Diagnostic Systems, Freehold, N.J.

1. Qualitative acid phosphatase assays may be run conveniently in microtiter plates.

2. Pipette 5 µl of extract supernatant in duplicate into plate wells.

3. At timed intervals, add 20 µl acid phosphatase substrate solution (prepared as per manufacturer's instructions) into each well.

4. Carefully float plate on the surface of water which is 37° C.

5. Permit the assay to proceed for 30 minutes. Terminate the reactions at timed intervals by adding 100 µl of the NaOH supplied with the assay kit.

6. Evaluate the results as positive, weak, trace or negative.

BGS determination

1. Label plate appropriately.

2. Aliquots of 10 µl of stain extracts are pipetted into the bottom of the appropriate microtiter plate wells. Questioned specimen extracts are analyzed in duplicate. Extracts of known group semen stains and cloth controls are run singly.

3. The horizontal rows of wells receive 20 µl of anti-A, anti-B or H-lectin. Pipette antisera and lectin directly into the drop of extract. Do not dribble down the side of the well.

The dilution of anti-A, anti-B or H-lectin used for this test must be determined in advance. See Appendix II.

4. The well contents can be mixed by gently tapping the corners of the plate on the edge of the lab bench.

5. Cover plate (to minimize evaporation) and permit to stand for 30 minutes at room temperature.

6. To the A test row, add 20 µl group A erythrocytes, 20 µl B cells to the B row and 20 µl O cells to the H row. Mix by tapping. Cover and let stand 30 minutes.

Each cell suspension is 0.5 percent in HBS-preparation described in Appendix I.

7. The plate is centrifuged in a GLC –2 for 5 minutes at 1500 rpm.

8. Place the plate on the tilt stand for at least 20 minutes.

9. Read the results by eye. The presence of BGS in an extract is exemplified by a smooth run of cells from the pellet, whereas the absence of BGS results in complete agglutination of cells which remain as a pellet.

10. Nuances in the results fall into several categories.

Occasionally, a pellet which is completely agglutinated will fall, intact, to the edge of the well during the tilt phase. Sometimes, agglutinated pellets will be seen to sag or bulge at the bottom edge. Such results are positive.

The bench mark for evaluating weak negatives is observation of the positive known cloth or filter paper

control. This control should be unequivocally positive. Any suggestion of running in this control compromises interpretation of all other samples.

Such is not the case always with questioned specimen cloth controls. Because this technique is extremely sensitive, one may observe weak negative reactions with some Q controls. It is believed that the test is detecting BGS indigenous to the garment (from perspiration) in these cases.

Appendix I—Preparation of Erythrocyte Suspensions

The microtiter plate system for BGS detection is carefully calibrated for use with 0.5 percent suspensions of indicator erythrocytes. Thus, the cell suspensions must be prepared accurately. Unfortunately, the time-honored fashion of diluting a certain volume of packed erythrocytes into a much larger volume of saline to obtain a specified cell concentration suffers from numerous inaccuracies. Red cell suspensions of known concentration can be accurately prepared by measuring the turbidity of the cell suspension.

The turbidity of a cell suspension is directly related to the concentration of cells present (Figure 2). Thus, by knowing the turbidity value for the 0.5 percent cell suspension we desire, it is a simple matter to measure the turbidity of a conventionally prepared suspension and, if necessary, adjust to the desired value. The following steps outline this procedure:

1. Wash the RBC three times with HBS.
2. Prepare the usual 0.5 percent suspension by pipetting 50 μl packed RBC into 10 ml HBS. Mix well.

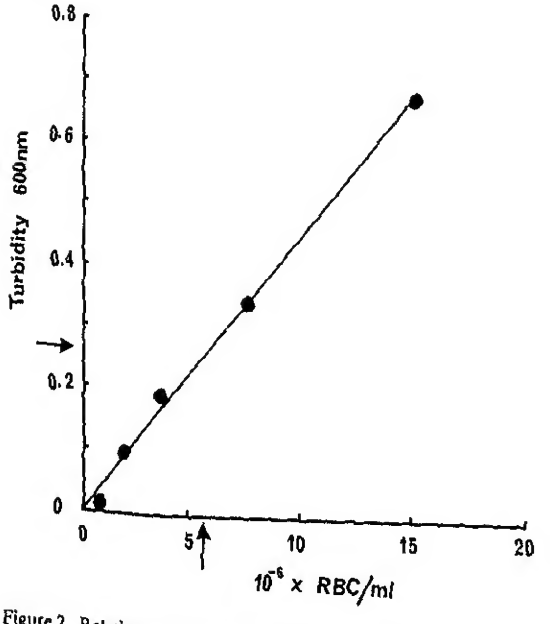

Figure 2. Relationship between turbidity and red blood cells.

3. Prepare a 1:10 dilution of the suspension by mixing 0.5 ml suspension with 4.5 ml HBS.
4. Determine the absorbancy of the 1:10 dilution at 600 nm using HBS as a blank.
5. A true 0.5 percent suspension diluted 1:10 will yield an absorbance of 0.26 ($A_{600} = 0.26$).
6. In practice one can accept an A_{600} between 0.23 and 0.29.
7. If the A_{600} is not within the specified range, you must do one of the following:

a. A_{600} greater than 0.29: the original 0.5 percent suspension must be diluted by a factor equal to

$$\frac{A_{600}}{0.26}$$

For example, A_{600} of the 1:10 suspension = 0.39

$$\frac{0.39}{0.26} = 1.5$$

Thus the original suspension must be diluted 1:1.5 (e.g., 10 ml suspension + 5 ml HBS)

b. A_{600} less than 0.23: the original 0.5 percent suspension must be concentrated by a factor equal to

$$\frac{A_{600}}{0.26}$$

For example, A_{600} of the $1:10 = 0.2$

$$\frac{0.2}{0.26} = 0.77$$

The 0.5 percent suspension must be spun, the liquid removed and the RBC resuspended in 0.77 times the original volume (e.g., 10 ml × 0.77 = 7.7 ml resuspension volume).

Appendix II—Titrations of Isoantisera and Lectin to Determine Their Agglutination Endpoints

The sensitivity of an absorption-inhibition test for soluble antigens is directly related to the concentration of antibody molecules present. Ideally, sufficient antibodies will be available in the assay to effect total agglutination of the indicator cells with few unbound antibodies remaining. As the number of excess antibodies increases, the sensitivity of the detection system will decrease. Thus, it is the objective of the antiserum and lectin titrations to determine the most dilute antiserum/lectin concentrations which completely agglutinate the indicator cells.

Once a particular lot of antiserum or lectin has been titered optimally, that calibration is relatively stable for the lifetime of the lot as long as the other assay variables remain unchanged.

To avoid repeated entrance into the stock solutions of antisera/lectin, which almost ensures microbiological contamination, working stock solutions of antiserum/lectin are prepared. It has been convenient to use 20X dilutions of these reagents as working stocks.

All 20X dilutions are prepared with THBS. 20X = 0.1 ml antiserum/lectin + 1.9 ml THBS.

Titration Protocols

1. Antiserum/lectin titrations are carried out with duplicate assays for each dilution. Since we are calibrating the test system for ultimate use with stain extracts, the volume in each test well normally occupied by extract must be replaced by HBS. Thus, each test well first receives 10 μl HBS.

2. Each well next receives 20 μl anti-A, anti-B or H lectin of the appropriate dilution (Table 1). You may use the same pipette tip to dispense all the dilutions of a given antiserum if you begin with the most dilute solution of reagent and pipette sequentially toward the most concentrated.

3. Add 20 μl of the proper indicator cell suspension (0.5 percent) to each well. Mix and let stand 30 minutes.

4. Centrifuge, tilt and evaluate as described.

5. The endpoint of an antiserum is defined as the greatest dilution which accomplishes total agglutination. For use in actual inhibition tests, one uses an antiserum dilution two or three steps back from the endpoint. As one's technical competence improves, one may utilize antiserum dilutions closer to the endpoint and achieve greater sensitivity.

Table 1. ISOANTISERA AND LECTIN DILUTIONS

Final dilution sought	*Actual dilution*	*Dilution of 20X*	*ml 20X*	*+ ml THBS*
Anti-A.				
600	240	12	0 05	0 55
700	280	14	0 05	0.65
800	320	16	0 05	0 75
900	360	18	0.05	0 85
1000	400	20	0.05	0 95
2000	800	40	0 02	0 78
3000	1200	60	0.01	0.59
4000	1600	80	0.01	0 79
Anti-B·				
700	280	14	0.05	0.65
800	320	16	0 05	0 75
900	360	18	0.05	0 85
1000	400	20	0 05	0.95
2000	800	40	0 02	0 78
3000	1200	60	0.01	0.59
4000	1600	80	0 01	0.79
5000	2000	100	0 01	0 99
H-Lectin:				
100	40	2	0 3	0.30
200	80	4	0 2	0 60
300	120	6	0.1	0.50
400	160	8	0 1	0.70
500	200	10	0.1	0 90
600	240	12	0.05	0.55
700	280	14	0 05	0.65
800	320	16	0.05	0 74

Appendix III—Secreted Blood Group Substances

The BGS which are elaborated into the body fluids of those individuals who are secretors are serologically indistinguishable from the BGS found on erythrocyte membranes. There are some biochemical differences in red cell and body fluid BGS; the former are glycolipid, whereas the latter are exclusively glycopeptide.

The secreted BGS are water soluble and composed of about 80 percent carbohydrate and 20 percent protein. Their molecular weights can range from 200,000 to 1,000,000. A, B and H antigens, when present in saliva or semen, are all found to be determinants on the same large molecule, for all antigens are coprecipitated by a monospecific antiserum to any one.

SECTION IV
PANEL DISCUSSIONS

INTRODUCTION TO PANEL DISCUSSIONS

There were two panel discussions held during this symposium. Generally, the panels were organized in the same manner. There were five forensic scientists on the panel in addition to the moderator. Each panel member had the opportunity to make a brief statement about the respective topic and then comments and/or questions were requested from the 150 attendees in the audience.

Panel Session I was titled "Laboratory Methods and Protocols Used in the Examination of Sexual Assault Evidence (Emphasis on Controversial Areas)." This session was taped and the moderator, Ken Konzak, summarized the discussion. That summary appears in this section.

Panel Session II was titled "Interpretation of Results in Sexual Assault Cases and Reporting Methods." This panel operated in a similar format except that a survey was conducted prior to the symposium by Richard Tanton. He prepared a ficticious set of data from a sexual assault, including a brief description typical of an officer's report. This information was mailed to 120 of those that were to attend the symposium. The interpretations and type of report from the 82 respondants were varied so Tanton has presented five that were typical of these responses. In Tanton's write-up are these five reports and the comments of individuals in the judicial system that were asked to evaluate these five reports.

As you read the reports you will notice very different styles from those responding in their content and length of report including interpretation. During the panel discussion it became apparent from those serving on the panel and those in the audience that many different reporting methods were used and some of the reasons were agency policy or personal preference of the examiner. Some individuals were of the opinion that all interpretations should be in the report while others were of the opinion that interpretation should be done in the court under testimony. If there was a consensus on this topic it was there was no majority, that there was an even distribution among panel members and in the audience as to style.

LABORATORY METHODS AND PROTOCOLS USED IN THE EXAMINATION OF SEXUAL ASSAULT EVIDENCE (EMPHASIS ON CONTROVERSIAL AREAS)

Panel Moderator: *Ken Konzak,* Montana Division of Forensic Science

Panel Members: *Jan Bashinski,* Oakland Police Department Laboratory
Marion Dorrill, Home Office Central Research Establishment
Keith Kelder, Toronto Centre of Forensic Science
Peter Martin, Metropolitan Police Forensic Science Laboratory
Robert Shaler, Office of the Chief Medical Examiner, New York

The discussions ranged from numerous areas of general agreement, particularly as to the nature of the problems, to significant differences in approaches to accommodate them. Early in the discussion, Jim Kearney (FBI) noted that it was difficult to compare results, particularly those concerning the levels of secreted substances, because we were constantly talking "apples and oranges" in terms of techniques, reagents and titers. No one, it was generally agreed, could make a valid correlation. Therefore, the disagreements in results and some so-called "aberrations" might be explained by different approaches or techniques.

The bulk of the discussions dealt with differentiating secretors from nonsecretors of A, B and H substances and observing their correlation, or lack of, with Lewis types. In the classical theory, individuals with Lewis (a+b−) red blood cells and saliva would be nonsecretors of A, B and H substances, while Lewis (a−b+) individuals would be secretors and Le(a−b−) could be either (c+d− being nonsecretors and c−d+ being secretors). Nature threw a wrench into this plan by allowing secretors to produce varying levels of Le(a) in their saliva (and other secretions) and, according to the work of Mary Gibbons (Oakland), by allowing nonsecretors to produce varying, though lower, levels of Le b substances. The Lewis a/b ratio present in secretions may apparently be a better predictor for levels of secreted substances than the red cell type. The interim guidelines used by the Toronto laboratory are shown in Table 1. As Patrick Lincoln pointed out in the discussion, it has been known for many years that there is a gray area between secretors with low ABH levels and high-level nonsecretors. Truly anomalous results, such as Peter Martin's "B" secretions from a known type "A" donor, still defy explanation. It is obvious, however, that some of the results described by some as aberrations are in fact reflections of genetics, such as A_2B or A_3B secretors with low levels of A substances or the Se^w gene in Asiatics predicted by Tsang Feller.

Table 1. INTERIM GUIDELINES FOR INTERPRETATION OF LEWIS RESULTS

	Interpretation
1. $\frac{Le^a}{Le^b} \leq 1$	Secretor origin
2. $\frac{Le^a}{Le^b} > 1$, by *at least* 2 dilutions	Nonsecretor origin
3. Le^a and $Le^b = 0$, i.e., Le (a−b−) or $\frac{Le^a}{Le^b} > 1$, by only 1 dilution	Inconclusive

A number of approaches were presented to limit the apparent abnormalities one sees. Keith Kelder cited work that showed that the distribution of genetic markers across a stain is not uniform. This makes an extraction technique essential for the analysis of secreted substances. Jan Bashinski cautioned that material should be saved or extracted in a manner to allow isozyme or other analysis as well as ABH typing. She also noted that manditory drying, especially of vaginal swabs, immediately after collection greatly improves the chances for isozyme typing. The presence of amylase in a semen stain mixture provokes concern over the possibility of modification of PGM, but this can normally be accounted for by evaluating the quality of the banding and by examining both conventional and subtyping gels. Likewise, several panel members

[illegible] provided the use of Lewis typing of blood standards to confirm the secretor status identified by absorption-inhibition (A/I) or A/I and absorption-elution (A/E). This is not, however, to the exclusion of the analysis of the saliva standard. All suggested Lewis typing to confirm nonsecretors. Lewis typing of stains, not saliva standards, may be indicated by aberrant results or, in some typing schemes, by the lack of correlation of A/I with A/E results. As noted by Marion Dorrill titration of the antigen, whether ABH or Lewis, can assist in the interpretation of the significance of the results (particularly in mixtures per Jan Bashinski) and eliminates the possibility of non-specific agglutination reactions from high protein concentrations. The typing schemes or sequences used by the laboratories represented by the panel are depicted in Figures 1-4.

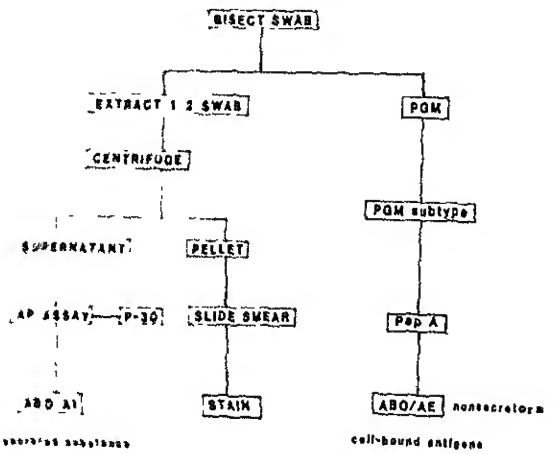

Figure 1. Sexual assault analysis scheme used by the Oakland Police Department.

Problems with cigarette butts and saliva stains were noted, especially by Keith Kelder, as requiring a Lewis a/b ratio for interpretation. Lip mucus and numerous sources of secretions we call saliva may be affecting these results by containing material produced at greater levels than elsewhere or material not produced solely in response to secretor genetics. Concern was also expressed by Peter Martin with reporting secretor type on materials such as cigarettes for which no conclusive identification of saliva can be made. It was noted by Marion Dorrill that saliva stains could be located by procion red amylopectin paper (Whitehead and Kipps 1975) or by the phadebas tablet gel technique (Willott 1974). An antihuman amylase has also been produced (Eckersall *et al.* 1981) which, while reactive to both salivary and pancreatic amylase (and therefore feces, sweat and, to a lesser extent, urine), did not react with animal salivas and "single disposition" sweat or urine stains. Saliva identification, they concluded, still remains a problem, particularly on cigarette butts.

Robert Shaler addressed some problem areas from his experience with the medical examiner's office. The problem of secretor status of cadavers, particularly when oral swabs are contaminated and the blood is not typeable in Lewis, could be approached, he noted, by analysis of salivary gland, stomach mucosa or testes extracts. Discussion also revealed examples of the use of bile and urine for this purpose. Perhaps of more immediate concern, the subject of Acquired Immune Deficiency Syndrome (AIDS) and other "evidence-borne" diseases was broached. Dr. Shaler recommended that whether or not AIDS is determined eventually to be a health risk (no medical or laboratory personnel have yet contacted it), all evidence should be treated as if it were contaminated with hepatitis. Ideally, the hands and face should be covered and a fume hood used. Hepatitis vaccines, as noted by George Sensabaugh, are currently felt to hold little danger of AIDS transmittal and are strongly recommended for serologists.

This meeting concluded with a discussion on sperm identification. Disagreement was voiced over whether two sperm heads would be sufficient for identification of semen when presently one intact sperm is required. The panel discussed these and other areas in a depth which may be more appropriately presented, due to its length, in the Crime Laboratory Digest or Forensic Serology News at a later date.

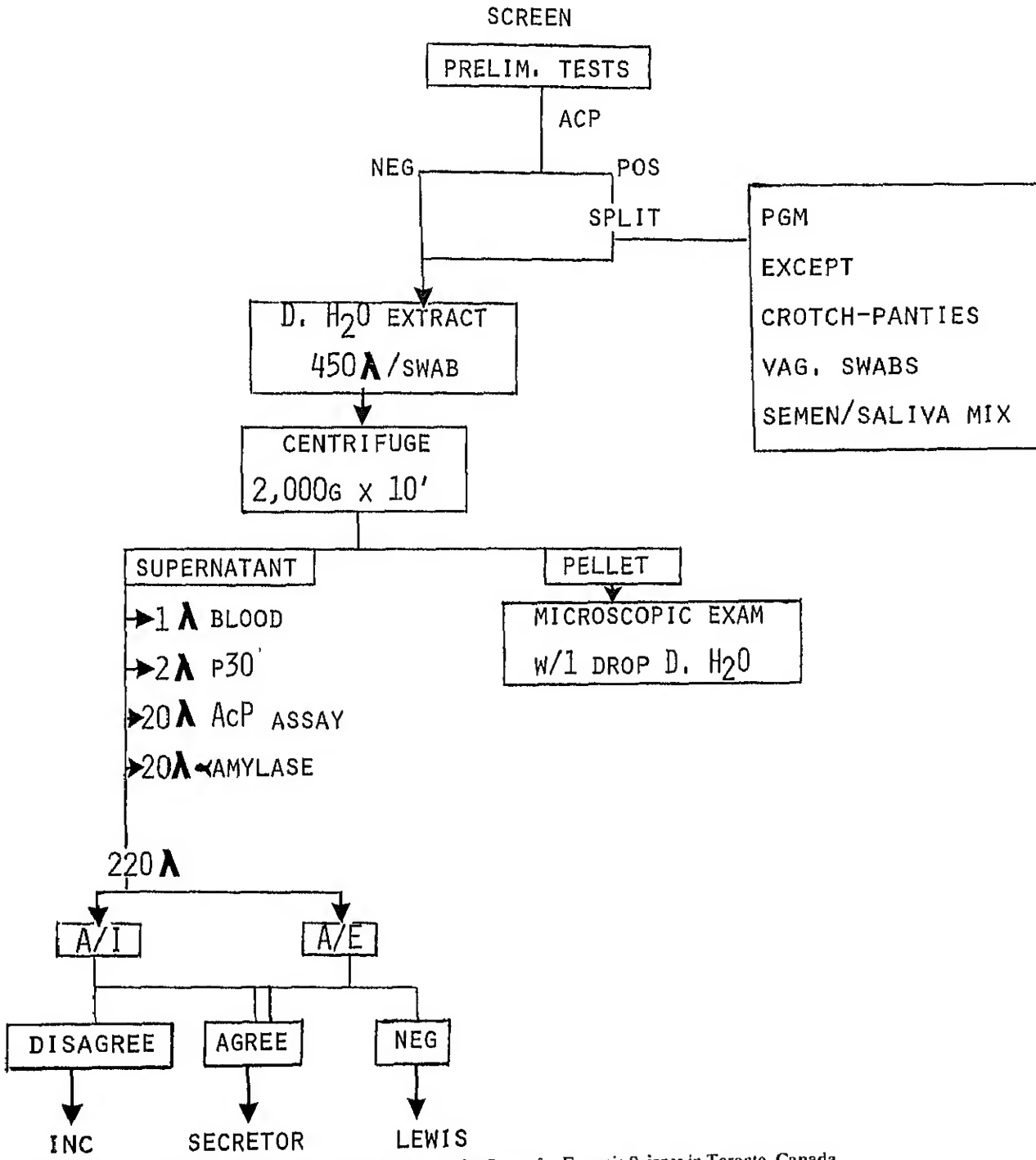

Figure 2. Sexual assault evidence analysis scheme used by the Centre for Forensic Science in Toronto, Canada.

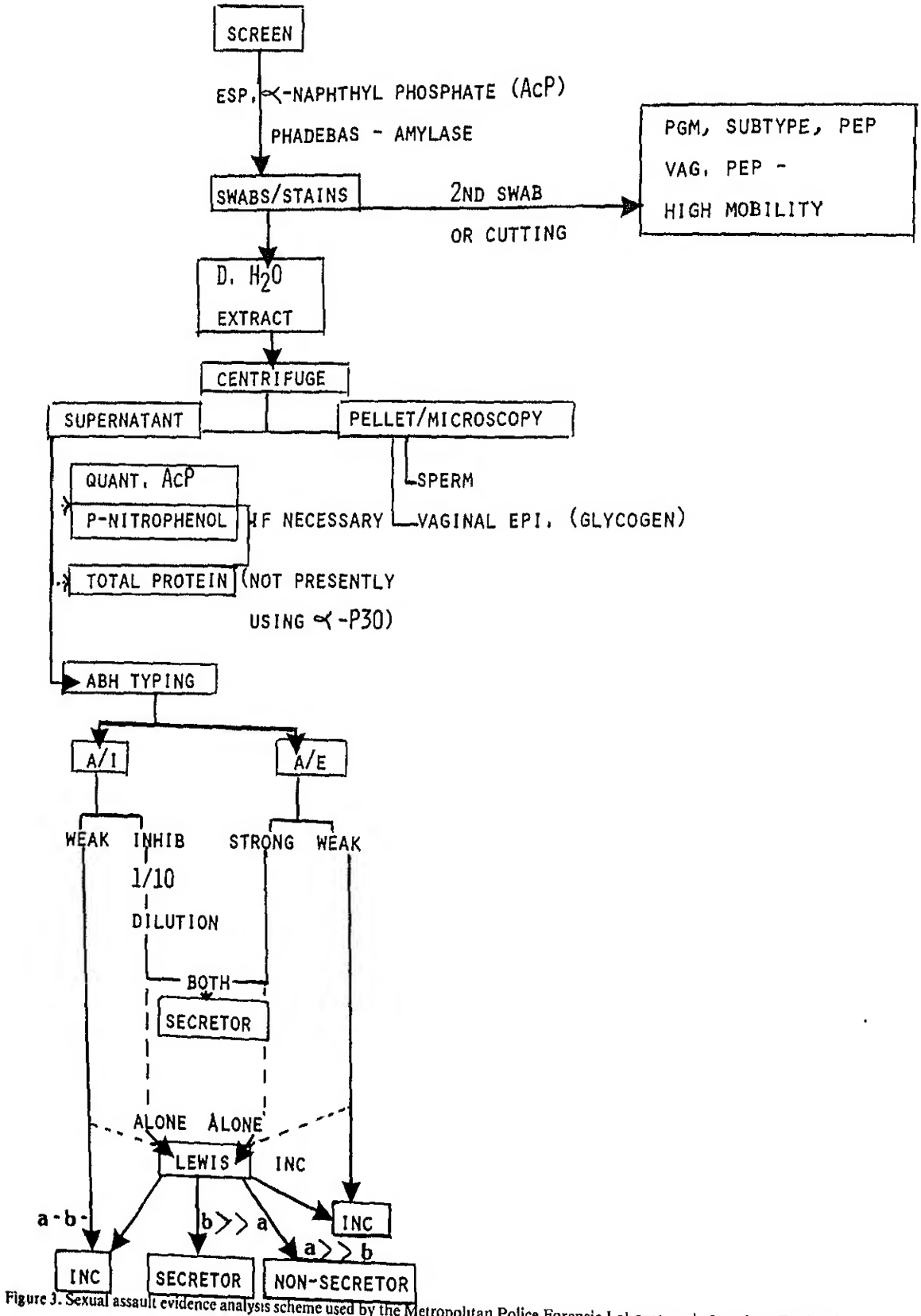

Figure 3. Sexual assault evidence analysis scheme used by the Metropolitan Police Forensic Laboratory in London, England.

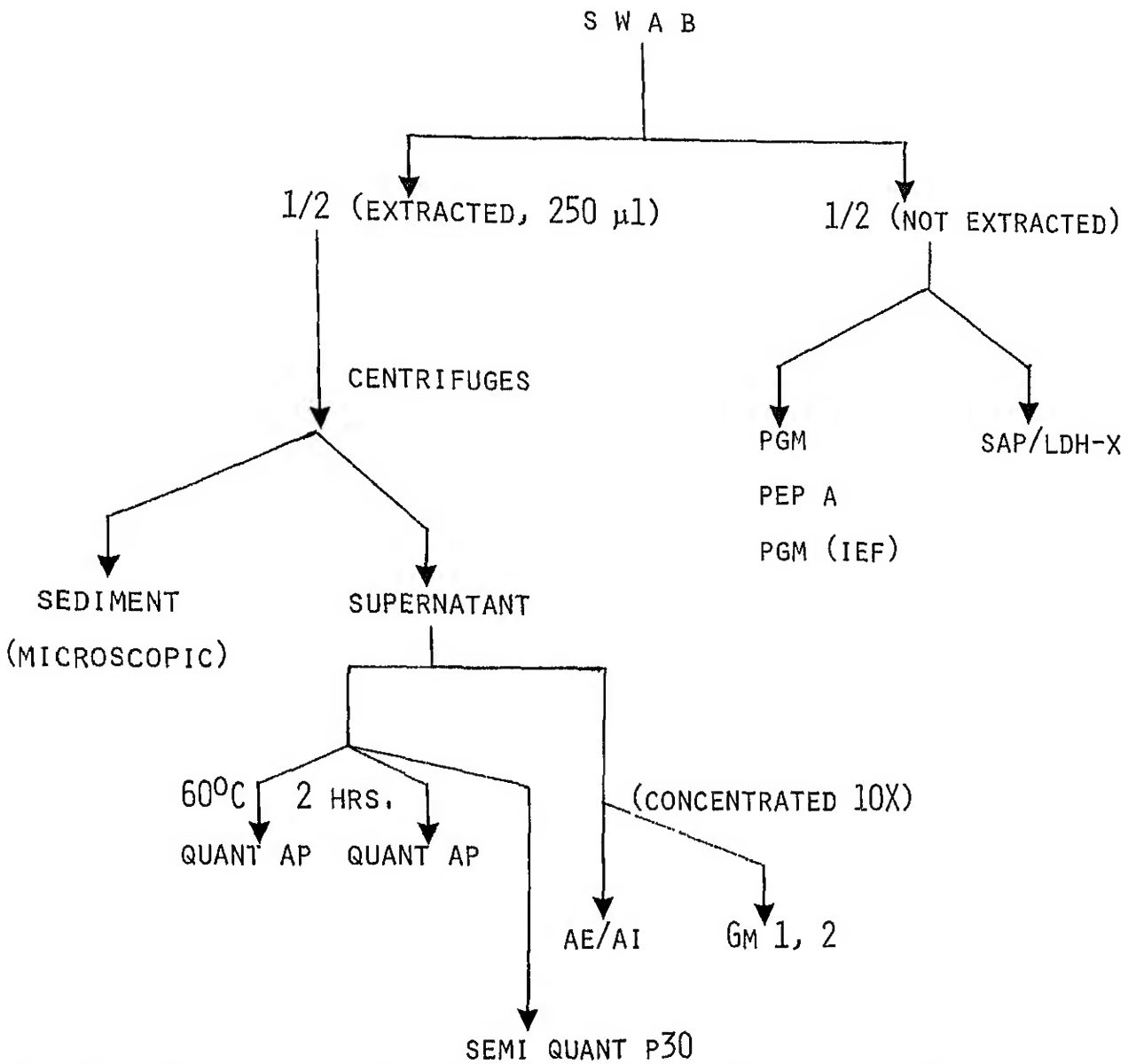

Figure 4. Semen analysis scheme (especially for dealing with cadavers) used by the New York Medical Examiner's Office

INTERPRETATION OF RESULTS IN SEXUAL ASSAULT CASES AND REPORTING METHODS

Panel Moderator: *Mark D. Stolorow,* Illinois Department of Law Enforcement

Panel Members: *Henry C. Lee,* Connecticut State Police Forensic Laboratory
Patrick J. Lincoln, London Hospital Medical College
David A. Metzger, Illinois Department of Law Enforcement
Robert P. Spalding, FBI Laboratory
Richard L. Tanton, Palm Beach County Sheriff's Crime Laboratory

A SEROLOGY REPORT WRITING EXERCISE

Conducted by *Richard L. Tanton*

The purpose of this exercise was to obtain a large sampling of serology reports based on common data. At this writing, I have 83 reports in hand (almost a 64 percent return—not bad) and promises of more to come. They vary considerably in form, content, parent agency (65) and place of origin (31 states, the District of Columbia, Canada, England, West Germany and Japan). This collection hopefully will encompass all the major philosophies and styles extant in forensic serology report writing today. It can and should be used as a resource document for further study and improvement of written communication in the field of forensic serology.

Toward that end, I have taken the liberty of characterizing these reports according to (1) length of pages, (2) presence of conclusions or interpretations, (3) tabulation of data, (4) organizational orientation—Results or Items and (5) presence of frequency data. These results are summarized in Table 1.

Table 1. CHARACTERIZATION OF REPORTS SUBMITTED

(1) Length of Report (pages): Shortest (1½ pages), Longest (3½ pages), Average (1.9 pages)

	Yes	*No*
(2) Interpretation or Conclusion	46 (55%)	37 (45%)
(3) Tabulation of Data	33 (40%)	50 (60%)
(4) Orientation—Results	54 (65%)	—
Items	29 (35%)	—
(5) Frequency Data	8 (10%)	75 (90%)

In addition to this, I selected five of the submitted reports which seemed to represent the major styles and content variations. I sent these five reports along with an explanatory memo (Appendix 1) to some investigators (5), prosecutors (3) and defense attorneys (4), and asked them to evaluate and rank the samples from 1 (the best) to 5 (the worst). In addition, I asked them to keep in mind these two facts: (1) this is a small, unsophisticated survey and (2) "recipient popularity" should not be the sole criterion for report evaluation. The numerical results are presented in Table 2. Additional written comments are presented in Appendix 2.

Since most of you were kind enough to send me your reports, I feel it only proper for me to give you a copy of my report on this exercise (see Appendix 3).

Table 2. NUMERICAL RESULTS OF REPORT EVALUATIONS

	Report (Average Score)				
Survey Subjects	*A*	*B*	*C*	*D*	*E*
Investigators (5)	4.4	2.1	2.0	2.7	3.8
Prosecutors (3)	4.7	1.3	2.0	3.0	4.7
Defense Attorneys (4)	4.8	2.0	1.0	3.0	4.3
Total (12)	4.6	1.9	1.7	2.9	4.2

significantly in both style and content. What I would like you to do is as follows:

(1) Read the case history below and Criminalists Comparison and Analysis Form (Figures 1 and 2).

(2) On each of the report examples write your comments on that particular example.

(3) Rank the report examples from 1 (the best) to 5 (worst).

Case History

The victim, Virginia Cherry, 16 W/F, was sleeping soundly when she was awakened by two white males who gagged her and forced her to submit to penile-vaginal intercourse. Both men ejaculated. After the assailants left, Ms. Cherry called the police. Several hours later, two white, male suspects were apprehended: Studds Macho, 26 years old, and Willie Climax, 22 years old. Ms. Cherry reported that her last prior intercourse had been 2 weeks ago.

Normally, I would like to give you plenty of time to thoroughly study these reports and reflect on your rankings and comments, however, I am under extreme time pressure. If you could complete this exercise within the next day or so, I would be eternally grateful. In fact, I may even consent to make some of my testimony understandable—no promises though.

Begging you in advance,
Richard L. Tanton

REPORT A

Subject: *Virginia Cherry* (victim)
Studds Macho (defendant)
Willie Climax (defendant)

Item: Submitted by *Richard L. Tanton*
Evidence delivered by mail on June 14, 1983.

Examination:

1. BLOOD from CHERRY
2. SALIVA from CHERRY
3. BLOOD from MACHO
4. SALIVA from MACHO
5. BLOOD from CLIMAX
6. SALIVA from CLIMAX
7. VAGINAL SWABS—Human semen stains present.
8. NIGHTGOWN—Human semen stains present.
9. BEDSHEET—Human semen stains present.
10. SMEAR SLIDE—Spermatozoa present.

Items Nos. 1 through 10 refer to Blood-Group Profile.

BLOOD-GROUP PROFILE

	PGM	*Le*	*ABO*	*Consistent with blood group*
1 Blood—Cherry	1	B	H	O
2. Saliva—Cherry			H	O
3 Blood—Macho	1	B	B,H	B
4 Saliva—Macho			B,H	B
5. Blood—Climax	2	B	H	O
6. Saliva—Climax			H	O
7. Vaginal swabs	2-1		A,H	A
8 Nightgown	2-1		A,H	A
9. Bedsheet	2		H	O

Disposition

Evidence returned to Palm Beach Sheriff's Crime Lab, West Palm Beach, Florida, by mail.

REPORT B

The following is a summary of the analytical findings:

Exhibit	*Item submitted*	*Findings*
1	Blood of Virginia Cherry	ABO = O, Lewis = Le (-) Le (+) (indicates a secretor in ABO), PGM = 1
2	Saliva of Virginia Cherry	H activity detected, consistent with Group O secretor
3	Blood of Studds Macho	ABO = B, Lewis = Le (-) Le (+) (indicates a secretor in ABO), PGM = 1
4	Saliva of Studds Macho	B and H activity detected, consistent with a Group B secretor
5	Blood of Willie Climax	ABO = O, Lewis = Le (-) Le (+) (indicates a secretor in ABO), PGM = 2
6	Saliva of Willie Climax	H activity detected, consistent with a Group O secretor.
7	Vaginal swabs from Virginia Cherry	Seminal material identified. A and H activity detected. PGM = 2-1
8	Nightgown of Virginia Cherry	Seminal material identified. A and H activity detected PGM = 2-1
9	Bed sheet from Virginia Cherry's bed	Seminal material identified H activity detected. PGM = 2
10	Vaginal smear from Virginia Cherry	Seminal material identified

Conclusions

The seminal stain on the bed sheet (Exhibit 9) could have originated from suspect Willie Climax. In addi-

tion, Willie Climax could have contributed part, but not all, of the seminal material identified on the vaginal swabs (exhibit 7) and nightgown (exhibit 8).

The seminal material on the bed sheet (exhibit 9) could not have originated from suspect Studds Macho. In addition, there is no evidence to indicate that the seminal material on the vaginal swabs (exhibit 7) or the nightgown (exhibit 8) originated wholly, or in part, from suspect Studds Macho.

REPORT C

Blood Grouping Report

Tests performed on blood and saliva samples from Cherry, Macho and Climax produced results which show that:

Cherry is group O secretor PGM 1
Macho is group B secretor PGM 1
Climax is group O secretor PGM 2

Vaginal swabs

Human semen was detected on these swabs, and reactions for group A secretor PGM 2 -1 were obtained.

These reactions could be due to secretions originating from Cherry mixed with semen of group A secretor PGM 2 -1 or group A secretor PGM 2. The A secretor and PGM 2 reactions must have originated from the semen and such semen could not have come from Climax as he is group O or Macho who is group B and PGM 1.

However, the possibility of there being, in addition, semen of group O of any PGM group, cannot be ruled out. Such individuals occur with a frequency of about 47 percent among unrelated white American males. Since Climax is group O secretor PGM 2, the possibility that his semen is present on these swabs cannot be excluded.

The results obtained do not provide any evidence for the presence of semen of group B secretor, Macho's group.

Nightgown

Stains of human semen were detected on this item and grouping tests showed that the semen staining gave reactions for group A secretor PGM 2 -1.

The group A activity could not have originated from either Macho or Climax who are group B and group O, respectively, and therefore, there is semen on this nightgown which could not have originated from either of these men.

The possibility of semen of group A or O of any PGM group also being present in this stain cannot be ruled out. About 89 percent of unrelated white Americans, including Climax, would be included in this category.

The results obtained did not provide any evidence for the presence of semen of group B secretor, Macho's group

Bedsheet

Human semen staining detected on this item gave reactions for group O secretor PGM 2

Semen of this combination of groups could not have originated from Macho since he is group B secretor PGM 1, but there is nothing against it having originated from Climax. About 2 percent of unrelated white American men would be expected to be of these same groups and could not, from the blood group point of view, be excluded as possible donors of this semen

Smear slide

Results obtained on this slide demonstrated the presence of semen.

Summary

These results provide evidence for the presence of at least some semen on the vaginal swab and the nightgown which could not have originated from either Macho or Climax. However, the possibility of there being, in addition, semen from Climax on these two items cannot be ruled out.

There is nothing against the semen staining on the bedsheet having originated from Climax, but this staining could not have come from Macho

REPORT D

This is a report concerning physical evidence examinations requested by your office. In any future correspondence regarding this case, please use the BFS case number appearing at the top of this report. If court testimony is required, please notify this office at least two weeks in advance whenever possible.

Summary

Sperm cells were detected on the glass slides (Item 10).

Semen was detected on the vaginal swabs (Item 7), nightgown (Item 8) and bedsheet (Item 9). Genetic markers different from the suspect MACHO were detected in all the semen stains. Genetic markers similar to the suspect CLIMAX cannot be eliminated as a

SHERIFF'S OFFICE
PALM BEACH COUNTY

☒ WEST PALM BEACH ☐ DELRAY BEACH ☐ BELLE GLADE

CRIMINALISTICS COMPARISON AND ANALYSIS FORM

EXAMINER: Crime Lab #S-1983

1 CLASSIFICATION	2 CRIME	CODE	3 GRID CODE	4 DATE	5 DAY OF WK	6 WEATHER	7 CASE NO
SEROLOGY				4/01/83	Fri.	N/A	83-0001

8 OCCURRED AT NUMBER STREET APT CITY	9 UNIT TAG NO	10 ZONE	11 DEPUTY NO	12 TIME DISP	13 TIME ARRIVED	14 TIME COMPL	15 ASSIST	16 RESIDENT ☐ NON RESIDENT ☐
1201 Prosperity Farms Road, Palm Beach Gardens,	FLA.		1618	12 noon	4-4-83	4-8-83	PBSO	

18A. AGENCY CODE	17 AGENCY OFFICER ASSIGNED	17A OFFICERS AT SCENE
	Det. Richard L. Tanton - PBSO Detective Bureau/Headquarters	

18 ☒ VICTIM ☐ SUSPECT	AGE	SEX	RACE	RESIDENCE ADDRESS	BUSINESS ADDRESS	PHONE
Virginia Cherry	16	F	W	1201 Prosperity Farms Road, Palm Beach Gardens, Fla.		622-0011
18 ☐ ARRESTED ☒ SUSPECT ☐ JUVENILE FULL NAME				RESIDENCE ADDRESS CITY STATE		PHONE
1) Studds Macho (26 w/m) transient				2) Willie Climax (22 w/m) transient		

20 TYPE OF ASSISTANCE (CHECK)

- ☐ CHEMICAL ANALYSIS
- ☐ OPTICAL ANALYSIS
- ☐ INSTRUMENTAL ANAL.
- ☐ CRIME SCENE
- ☐ PHOTOGRAPHY
- ☐ FINGER PRINT
- ☐ POLYGRAPH
- ☐ COMPUTER SEARCH
- ☐ MIRACODE SEARCH

20A. ANALOG (CHECK)

- ☐ DANGEROUS DRUGS
- ☐ NARCOTIC
- ☐ BEVERAGE
- ☐ BALLISTICS
- ☐ TOOL MARK
- ☐ TOXICOLOGY
- ☒ SEROLOGY
- ☐ PAINT
- ☐ HAIR & FIBER
- ☐ EXPLOSIVES
- ☐ OFFICE
- ☐ FIELD
- ☐ MOVIE
- ☐ STILL
- ☐ VIDEO TAPE RECORDING
- ☐ BLACK AND WHITE
- ☐ COLOR
- ☐ PROCESSING
- ☐ MULAGE
- ☐
- ☐

21 EVIDENCE (LIST)

1) Sample of Cherry's blood
2) Sample of Cherry's saliva
3) Sample of Macho's blood
4) Sample of Macho's saliva
5) Sample of Climax's blood
6) Sample of Climax's saliva
7) Vaginal swabs
8) Nightgown
9) Bedsheet
10) Smear slide

(Interpretation - continued on page 2)

EVIDENCE SENT TO _____

EVIDENCE SENT BY _____

22 REMARKS

Interpretation

Either all, or part, of the semen found on the vaginal swabs (7) and the nightgown (8) was contributed by an "A" - secretor male. Approximately 1 out of every 3 South Florida males fall into this group. Also, either all, or part, of this same semen was contributed by a PGM 2-1 or 2 male. Approximately 4 out of every 10 South Florida males fall into this group.

Neither Macho nor Climax are "A" - secretors and thus could not have contributed all the semen found on these items. Climax could have been a partial contributor, but there is no evidence that Macho contributed any semen to these items.

The semen stain found on the bedsheet (9) was contributed all, or in part by either an "O" - secretor or a non-secretor, PGM-2 male. Approximately 1 out of every 30 South Florida males

23 REPORTING DEPUTY'S SIGNATURE: Jay T. Pintacuda, Chief Chemist

24 CRIME SCENE TECH CALLED ☐ YES ☐ NO — NAME 1 _____ 2 _____

25 STATE TWX MSG NO _____ LOCAL TWX MSG NO _____

26 NCIC ☐ YES ☐ NO

Figure 1. Page 1 of Criminalistics Comparison and Analysis Form used by Sheriff's Office, Palm Beach County, Florida.

SEROLOGY

victim: Virginia Cherry
suspects: 1) Studds Macho
2) Willie Climax

Crime Lab #S-1983
PBSO Case #83-0001

Interpretation (continued)

fall into this group. Climax falls into this group and thus could have been the sole contributor of semen to this stain. Conversely, Macho could not have been the sole contributor of this stain, and, as in the other stains, there is no evidence of any semen contributions from hism.

Results

A) Individual Standards - Cherry's, Macho's, and Climax's blood and saliva sample contained the following blood factors:

	ABO	Le	PGM
1) Cherry's blood	O	B	1
2) Cherry's saliva	O		
3) Macho's blood	B	B	1
4) Macho's saliva	B		
5) Climax's blood	O	B	2
6) Climax's saliva	O		

B) Semen-Stained Items - Human semen stains were found on the vaginal swabs (7), the nightgown (8), and the bedsheet (9). These semen stains contained the following blood factors:

	ABO	PGM
7) Vaginal swabs	A,(O)	2-1
8) Nightgown	A, (O)	2-1
9) Bedsheet	O	

Spermatozoa were found on the smear slide (10).

Richard L. Tanton, Serologist

partial contributor to the genetic markers detected on the vaginal swabs (Item 7) and the nightgown (Item 8)

Evidence

The following items of evidence were received in the laboratory:

Item 1—Cherry's blood
Item 2—Cherry's saliva
Item 3—Macho's blood
Item 4—Macho's saliva
Item 5—Climax' blood
Item 6—Climax' saliva
Item 7—Vaginal swabs
Item 8—Nightgown
Item 9—Bedsheet
Item 10—Smear slide

Results

Item	*Description*	*ABO*	*Lewis*	*PGM*	*Semen*	*Sperm Cells*	*ABH*
1	Cherry's blood	O	b +	1 -1			
2	Cherry's saliva						H
3	Macho's blood	B	b +	1 -1			
4	Macho's saliva						B, H
5	Climax' blood	O	b +	2 -2			
6	Climax' saliva						H
7	Vaginal swabs			2 -1	+		A, H
8	Nightgown			2 -1	+		A, H
9	Bedsheet			2 -2	+		H
10	Slides					+	

Note

The ABH type A activity cannot be attributed to either Cherry, Macho or Climax and must have originated from some other unknown source.

REPORT E

Exhibits 1 and 2

The liquid blood sample identified as having come from "Virginia Cherry" was found to be of blood group "O", and the enzyme phosphoglucomutase type 1 (PGM -1). The saliva standard (Exhibit 2) from "Virginia Cherry" gave reactions characteristic of an "O secretor."

Exhibits 3 and 4

The liquid blood sample (Exhibit 3) identified as having come from "Studds Macho" was found to be of blood group "B", and the enzyme phosphoglucomutase type 1 (PGM -1). The saliva standard (Exhibit 4) from "Studds Macho" gave reactions characteristic of a "B secretor."

Exhibits 5 and 6

Semen was identified on the vaginal swab (Exhibit 7) and the victim's nightgown (Exhibit 8) by the presence of spermatozoa, the male reproductive cells. Grouping tests indicate the presence of both the "A" and "O" blood group factors, and the enzyme phosphoglucomutase type 2 -1 (PGM 2 -1) on each of these exhibits.

Exhibit 9

Semen was identified on the bedsheet (Exhibit 9) by the presence of spermatozoa, the male reproductive cell. Grouping tests indicate the presence of the blood group "O" factor and the enzyme phosphoglucomutase type 2 (PGM 2).

Exhibit 10

Semen was identified on the vaginal smear slide (Exhibit 10) by the presence of spermatozoa, the male reproductive cells.

APPENDIX 2

Comments—(I =Investigator, P =Prosecutor, D =Defense Attorney)

Report A

1. No conclusions. Too complicated for layman. (Investigator would have to find writer and get things straight—poor (I)
2. I don't understand this report—bad (P)
3. No conclusions (I)
4. This says absolutely nothing about defendents (D)
5. Too difficult for layman to interpret (P)
6. Have no idea what means (D)
7. No conclusions (D)
8. Report lacks conclusions of serologist . . . very important . . . seems incomplete (P)

Report B

1. Too complicated for investigator (I)
2. Good—makes some sense (P)
3. Eliminated Studds Macho as suspect (I)
4. Somewhat unclear (D)
5. Good for analyzing data (P)
6. Good (D)
7. Very Good (D)

Report C

1. Some help, but still complicated (I)
2. Still doesn't make much sense (P)
3. Confusing—but like percent groupings (I)
4. (Chart would help), but report is quite clear (D)
5. The best, but combining B and C would be best (D)
6. Good simple analysis (D)
7. Too much narrative, scientific data not presented well (P)

Report D

1. Better concentrate more on Climax than Macho, but confusing in the end (I)
2. Bad—confusing to me (P)
3. Not bad—but need to explain "ABH Type A" (I)
4. (Does this) mean Macho can be eliminated? (D)
5. Too complicated (P)
6. Have no idea what this means. Summary is slightly helpful (D)
7. Chart is simple and direct, but meaning less to uninitiated (D)
8. Don't like the order. I prefer to see what items were received . . . first (P)

APPENDIX 3

Comments—I =Investigator, P =Prosecutor, D =Defense Attorney)

Report E

1. Gives better insight (I)
2. Confusing (P)
3. Items submitted not listed-no conclusion (I)
4. Does not incriminate or exculpate defendents (D)
5. Pits (P)
6. Interesting, but this one doesn't tell us what all this means in Engish. Conclusions would be helpful. Deposition would be needed to explain. (D)
7. No interpretation of findings, ergo, almost worthless in absence of further discovery by attorney (D)
8. This report doesn't tell me what I want to know, which is, could either of the suspects have deposited the semen on the bed linens . . . (etc.) (P)

Some Especially Confusing Terms

1. "Genetic markers"
2. "Consistent"
3. "Antigenic activity"
4. "Antigen"
5. "Blood Group Substance"
6. All types of double negatives—"cannot be eliminated"

SECTION V
SPECIAL PRESENTATION

SEX OFFENDER PROFILING BY THE FBI: A PRELIMINARY CONCEPTUAL MODEL

Park Elliott Dietz
University of Virginia
Charlottesville, Virginia

In comparison with the growing sophistication of serologic and immunologic methods used in the investigation of sexual assaults, behavioral techniques are in a primitive phase of development. This is largely a reflection of the vagaries of the human psyche. Not only is the behavior we seek to classify or measure subject to poorly understood sources of variance, but the human minds that are most often relied upon to do the classifying and measuring are poorly calibrated and unreliable instruments. Perhaps the most pertinent illustration of this difference between biological and behavioral measures is that under ideal conditions, rapists can now be more accurately identified by the biological properties of their semen than by an eyewitness to the rape (see Loftus 1979 and Yarmey 1979, for analyses of the failings of eyewitness identification), though jurors and judges are reluctant to believe so.

Despite many limitations, behavioral techniques have contributed to the detection of many offenders, as Robert Hazelwood (this volume) has so dramatically illustrated. The present paper is intended to give an observer's account of how it is that members of the Behavioral Science Unit of the FBI Academy develop criminal personality profiles in sex offense cases. In so doing I draw upon certain behavioral science principles in an effort to evaluate the conceptual and theoretical status of offender profiling.

THE PROCESS OF SEX OFFENDER PROFILING

In order to facilitate his adultery with the wife of Uriah the Hitite, King David sent Uriah to the forefront of battle so that he would be killed. After Uriah's death and the birth of a child to King David and Uriah's widow, the prophet Nathan informed King David that there was adultery in his Kingdom. When the King insisted that Nathan identify the culprit, Nathan responded, "Thou art the man" (II Samuel, Chapter 12, verse 7). In accusing the King of adultery, Nathan did not have the benefit of a confession, eyewitness testimony or semen typing.

Modern efforts to detect unknown offenders draw on all available investigative information and, increasingly, on available information about human behavior (particularly that concerning similar offenses and offenders). These form the basis for generating hypotheses about the person or persons who committed the offense under investigation. Within the FBI, these efforts have come to be known as "psychological profiling" or "criminal personality profiling."

The activities now known as profiling have a colorful history. Among their many antecedents are the masterful inferences of fictional detectives, most notably Sherlock Holmes, who also anticipated developments in serology, identification of tobaccos, interpretation of powder markings from the discharge of firearms and questioned documents examination (see Berg 1970 for examples). Those who have attempted to solve crimes have no doubt always practiced variants of profiling (with mixed results), but the first published compilation f profiling stories may have been Brussel's (1968) Casebook of a Crime Psychiatrist.

The confirmation of Brussel's hypotheses about "the Mad Bomber" and other cases has encouraged investigators whose conventional methods have proved fruitless to consult psychiatrists (usually just before or after consulting psychics). As a rule, however, psychiatrists, psychologists and other mental health professionals are poorly equipped for this task (e.g., the survey results reported by Kiel 1965), since most know little or nothing about crime (and even those who do have generally learned what they know entirely from examining or treating those mentally disordered offenders who have been caught).

The Boston Strangler investigation provides an example of the misguided efforts that can result from the well-intended advice of otherwise competent mental health experts. Police officials consulted a university-based team of psychiatrists and psychologists, asking for assistance in finding the Strangler. This team is said to have suggested that the murders were being committed independently by two individuals, one of whom was homosexual. The police consequently devoted enormous time and manpower to their investigation of Boston's homosexual community, while the heterosexual murderer continued to strangle women

(Porter 1983). In a symposium about public medicolegal consultation (McGarry *et al.* 1968), Kenefick, the psychiatrist who coordinated this team, described the ethical problems that arose as he was asked to interview a succession of suspects and the problems posed by political machinations, but does not mention how the team formulated its hypotheses or what they were[1].

Today the major center for profiling criminal offenders is the Behavioral Science Unit at the FBI Academy. Pioneering work has been done by a succession of Supervisory Special Agents, some of whom have since retired or moved on to positions elsewhere in the Bureau, and others of whom continue to develop, apply and teach profiling. Profiles developed in the unit have often been confirmed by subsequent identification of the offender. Many success stories and a consumer satisfaction survey indicating that many police departments receiving profiles find them helpful have generated great enthusiasm. Nonetheless, the unit is aware that the accuracy of profiling has not yet been measured.

Based on observations of members of the Behavioral Science Unit engaged in the work of profiling, I have developed a tentative conceptual model of their profiling strategy. For the most part my observations have been unsystematic and have occurred in the course of discussions about cases.

The tentative conceptual model of profiling is shown schematically in Figure 1. The profiler engages in a five-stage process, each of which is associated with a checkpoint. He begins by acquiring information about the case (Data Assimilation), and then tentatively determines the sequence of observable events that took place (Behavioral Reconstruction), why the participants behaved as they did (Motivational Hypotheses), what kind of person would be motivated to behave in this way (Typological Hypotheses), and what characteristics an individual who committed this offense in this way and for these reasons would be expected to have (Attributional Hypotheses).

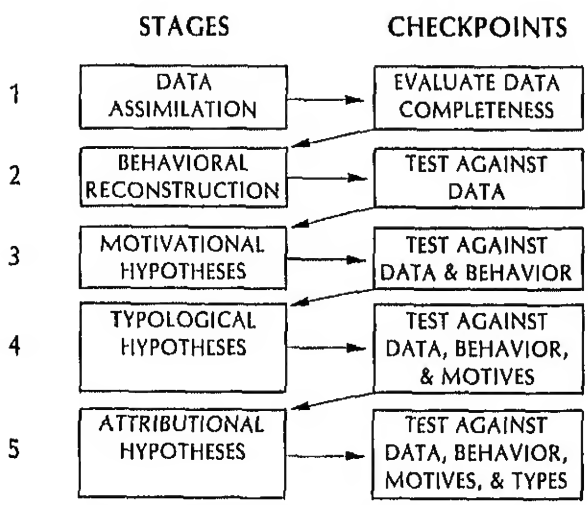

Figure 1. The five stages and checkpoints of offender profiling.

[1]It should be noted that Brussel (1968) reports having attended a meeting of the team at which he advanced the view that one man was committing the Strangler murders. It is clear from Brussel's hypotheses that he regarded the killer as heterosexual.

At each stage, the profiler checks his working hypotheses against the data and the hypotheses formulated in preceding steps. These "checkpoints," as they are referred to in Figure 1, are not actually discrete phases, but are a more continuous process in which the profiler seeks to ensure that his evolving hypotheses are consistent with the factual evidence and the overall set of hypotheses that he is constructing. He revises his thoughts as he proceeds, and he often leaps ahead to tentative attributional hypotheses, for they are the end product sought.

Once the attributional hypotheses are fully formulated, some of them are reported as the offender profile. The purpose of the profile is to guide the investigators. For that reason a high premium is placed on observable attributes that could narrow the field of suspects under consideration and characteristics that suggest routes of investigation.

In the following text, the five stages are considered in greater detail. Where appropriate, a critical comment is included on the type of analysis involved in that stage and the way in which this resembles or differs from related types of analysis used by behavioral scientists for other purposes. After all five stages have been described, they are illustrated with an example of a lust-murder case created from information in a paper by Hazelwood and Douglas (1980).

Stage 1: Data Assimilation

The first step toward developing a profile is the review of any and all investigative information available. Materials reviewed include photographs of the crime scene and surrounding area, autopsy photographs, victim and witness statements, autopsy, laboratory and other investigative reports, complete background information on the victim, information about the area in which the offense occurred and a map showing the crime scene and the victim's last known location. The profiler is not given information about any suspects. In sexual assault cases in which the victim remains alive, her or his minutely detailed account of everything that was said or done often proves to be

the most important source of information. Communications to the victim before or after the offense, such as telephone calls or letters, photographs, sketches or items of clothing received through the mail or left at her home or place of work are all of critical importance. What the offender takes with him is just as important as what he leaves The taking of valuables, souvenirs or trophies is each subject to a different interpretation.

All of these sources of information, including whatever aspects of the investigation have proved fruitless, are studied and taken into account. In the course of studying the data base, the profiler inevitably begins to formulate hypotheses and to test them against the facts as they unfold. In this process, some ideas are discarded as disconfirmatory evidence is encountered, while others are strengthened by confirmatory evidence. At each subsequent stage in the process, evolving hypotheses are again tested against the data base. Often an otherwise attractive set of hypotheses has to be discarded when a piece of evidence is recalled or newly discovered that does not fit the hypotheses. When this occurs, the profiler returns to the data base and repeats the process.

Some forensic psychiatrists engage in a similar process of data assimilation in conjunction with their evaluation of defendants. They usually have a great deal of information about the defendant, but too often lack information about the crime itself or the victim. In June 1983, I heard a psychiatrist testifying for the defense in a mass murder case say that he did not look at any scene photographs, autopsy reports, witness statements, or police reports because he feels he must rely solely on what the defendant tells him. On cross-examination he went so far as to say that if the evidence in the case were to prove that the defendant lied to him about everything, it would not change his opinion that the man was legally insane!

Stage 2: Behavioral Reconstruction

The second stage consists of establishing the sequence of events before, during and after the offense[2]. Even when there is no living witness, it is often possible to arrive at a clear picture of how the offender and victim encountered one another, whether there was cooperation between them up to a particular point, whether the offender brought a weapon or other preparations (e.g., rope, adhesive tape, handcuffs) with him, whether he attempted penetration and into which orifices, whether he ejaculated, whether the victim was conscious, semiconscious, unconscious or dead at the time of various injuries he inflicted, what efforts he made to conceal his identity or the crime, what he took or did not take with him, and how he departed. This is by no means an exhaustive list, but it illustrates that there are many components to a sexual assault, each of which needs to be taken into account in attempting to reconstruct the sequence of behavioral events.

As the profiler reconstructs the events, he checks tentative formulations against the data base, revising and rearranging until he finds a reasonable sequence that fits all the known facts. Here, as at each subsequent stage, there is a risk of becoming overly committed to a formulation that leaves some pieces of evidence unexplained or that is only one of several formulations that fit the facts equally well.

Behavioral reconstruction is analogous to what the forensic pathologist and forensic psychiatrist should each try to do in their medicolegal evaluations. Surprisingly, little has been written that might guide practitioners of forensic medicine in these efforts, and they are not necessarily trained or experienced in trying to reconstruct events from the available data. These have become important components of my evaluation of criminal cases and of civil cases involving violence (e.g., accidental death benefits cases or malpractice cases claiming the physician negligently caused or allowed a patient's suicide or violence toward others). What I have learned about behavioral reconstruction, however, I have learned by studying criminology and forensic pathology and by consulting with the Behavioral Science Unit, not from conventional studies in medicine, psychiatry, psychology or sociology.

Elsewhere we have given examples of behavioral reconstruction from the investigative evidence in sexual fatality cases in which the manner of death is equivocal (Hazelwood *et al.* 1982; Hazelwood *et al.* 1983). Behavioral reconstruction should be distinguished from the psychological autopsy (Farberow and Shneidman 1961; Litman *et al.* 1963; Tabachnick *et al.* 1966) and the psychiatric autopsy (Bendheim 1979), which are approaches to assessing the motivation, mental state or personality characteristics of a decedent. Both psychological and psychiatric autopsies are directed more toward the types of hypotheses sought in the last three stages of profiling (particularly Stage 3) than toward the type of objective description of behavioral events which is the goal of behavioral reconstruction.

[2]The dissection of crimes and violent acts into pre-offense, offense and post-offense phases has been suggested as an aid in developing measures for the control of crime-related injuries (Dietz 1978) and for the psychiatric evaluation of violent patients (Rada 1981), each of which tasks employs behavioral reconstruction for a purpose different from that in profiling. Such a dissection is useful here, too, to organize the raw data, as an outline for the reconstructed events, and to remind the profiler to consider each phase.

Stage 3: Motivational Hypotheses

The third stage comprises the formulation of motivational hypotheses to account for the reconstructed behavioral sequence. The profiler seeks to explain what the participants were feeling and thinking that led them to behave as they did. Indeed, Ault and Reese (1980) went so far as to say that motive is "the primary psychological evidence" for which the profiler is looking. Examples of motivational hypotheses in sexual assault cases are that the offender tied up his victim prior to penetration in order to feel that he had complete control over her, that the offender forced the victim to fellate him after anal intercourse in order to humiliate and degrade her, that the offender arranged brassieres obtained from the victim's bureau in a ring around her on the floor in order to enhance his arousal in accordance with a pre-existing fantasy, or that the offender stabbed and mutilated the victim before penetration or masturbation in order that her pain or blood could stimulate him to erection.

It should be understood that such motivational hypotheses are psychological fictions used as one step in solving a problem. Experimental psychologists have trouble enough defining and measuring such apparently simple motives as the hunger or thirst of a white rat, let alone the complexities of human motivation. Analogous fictions employed in other fields are the imaginary numbers of the mathematician, the dummy variables of the statistician, the rational man of the economist or the free will of the jurist. Like motives, these fictions are useful in helping us feel as if we have explained some set of observed relationships. But motives do not have a tangible existence with measurable physical properties any more than do imaginary numbers, dummy variables, rational men or free wills.

It is also worth noting that motive, strictly defined, is not an element of any criminal offense (LaFave and

than if we hypothesize motivation from the eviden
available. This is because we, like the offender, choo
motivational labels from whatever vocabulary is ava
able to us and impute them to the offender as if th
were something inside him. When A says that B's crir
was motivated by revenge or anger or lust, he ascrib
these internal states to B to explain B's behavior, and
is the same when it is B who makes these claims abo
himself. Neither A nor B can measure B's motives, b
both seek to make sense of B's actions by inferri
some internal state that caused B to act as he did.

In a sophisticated analysis of this issue, the sociol
gist C. Wright Mills (1940) argued that motives are n
fixed elements within an individual, but rather t
terms with which social actors, including the individu
interpret his conduct. From Mills' standpoint, t
formulation of motivational hypotheses is motiv
mongering, whether used to explain why the bo
went home early, why the patient is sexually arous
by the sight of baby carriages or why the offend
acted as he did.

As hinted at in the preceding sentence, psychiatris
particularly psychoanalysts, are among the most pr
lific motive-mongerers (as Mills observed). For e
dence of this one need merely read Abrahamser
"Motivation of Crime" (1946) or psychiatric pape
with titles such as "Notes on Motiveless Murde
(Podolsky 1956), "Murder by Adolescents w
Obscure Motivation" (Stearns 1957) or "Offens
with No Apparent Motive" (Finkelstein 1968). W
the offender on his couch, the psychoanalyst c
always "find" multiple motives, but even wh
these motives are believable and seem to "explai
the actions, it will never be known whether whate
is meant by the motives was present and of cau
significance at the time of the offense.

In the case of sexual offenses, it is interesting
compare what one psychiatrist says about moti
with what one sociologist reports that offenders
about their motivations. According to the psychiatr
a man with considerable clinical experience with sex
offenders, offenders can become aware only of th
among their motives that are acceptable to their c
science. Their true motives, discoverable only
intensive psychiatric and psychological examinati
are their contempt for themselves as men, their perc
tions of themselves as failures, their concern with v
ning the approval of others and their overly high ide
The rapist seeks warmth, approval and accepta
from his victim; the incestuous father is trying to c
vince himself that those closest to him can love h
the molester of little boys lavishes on them the lov
wished he had received from his parents (Roth 19
This is a psychoanalytic view, that motives lie in

unconscious and can only be discerned by those who are specially trained for such work and who conduct an intensive clinical examination.

According to the sociologist, whose data sources are published case reports and biographies, court transcripts and interviews with prisoners, sex offenders most often blame factors outside their control for their offenses. These explanations are a breakdown in mental functioning (e.g., "something just comes over me—I have a blackout"), an inner impulse that compels him to act against his will or defective social skills (e.g., "I was trying to ask her to come out with me"). They also attribute their behavior to provocation or seduction by the victim, a desire for special experience, a desire to frighten or hurt, or a refusal to accept the restraints of society. Magistrates were more likely to believe the explanations based on a breakdown in mental functioning, a compelling inner impulse or defective social skills, than they were to believe the explanations in which the offender took partial or full responsibility for his actions. Moreover, other prisoners were more accepting of sex offenders who claimed not to have known what they were doing at the time of the offense (Taylor 1972). These observations suggest that rewards are available to those who blame their offense on forces outside their control.

It should be clear that there is no way to measure the validity of motivational hypotheses. They can be judged only by the degree to which they appear to make sense of the factual evidence and the behavioral reconstruction and by their usefulness in moving on to the fourth stage.

Stage 4: Typological Hypotheses

By the time the profiler has developed motivational hypotheses to account for the sequence of behavioral events, he has begun to postulate what type of person it was who did these things. For example, he will hold a tentative hypothesis as to whether the offender was organized or disorganized, a dichotomy that has been emphasized by Hazelwood and Douglas (1980).

The typologies employed in profiling are specific to the type of offense under investigation. The typologies used have been developed specifically for this purpose. Conventional literature in criminology, though consulted, proves of little use for profiling. Thus, the categories used do not always resemble more familiar nomenclature. The originality, specificity and use of a profiling typology will be illustrated below with the example of lust murder.

Classification of offenders into a typology (a set of preconceived, fixed categories) has a long and controversial history in criminology. Perhaps criminal typologies arose as a consequence of the biological and medical training of influential criminologists of the last century, such as Lombroso and Krafft-Ebing. Impressed by the achievements of Linnaeus in the 18th century and Darwin in the 19th in developing natural typologies (i.e., taxonomies) of biological specimens, medically trained criminologists tended to seek similar natural, biologically based taxonomies of criminal specimens. Be that as it may, the use of categories has great appeal to those who value parsimony. More important, the study of complex phenomena such as criminal behavior requires some reductionist maneuvers, including efforts to classify offenders, offenses and criminogenic processes (Dietz 1977).

Although they form a primitive level of measurement, nominal categories are of considerable value if they fulfill certain criteria. They should, among other things, be mutually exclusive and exhaustive. Their value for a particular purpose varies directly with the number and strength of each category's correlations with other variables. The greater the number and strength of its correlates, the more a category will appear to "explain" and the more accurate will be one's predictions of the attributes associated with a member of that category.

A typology accompanied by no decision-rules that allow one to classify a case is of no use. An example of this failing is Cassity's (1927) classification of pedophiles into two groups: those influenced by the trauma of weaning and those whose identification with the mother caused them to become rivals to the father. Even if a psychoanalyst could measure these concepts, it is doubtful that they are mutually exclusive.

A typology that uses overlapping categories or categories that together do not encompass all cases is illogical and confusing. Consider, for example, two categories defined by Ellis *et al.* (1954). The category "major sex offense" included "sexual assault, rape, incest, sex relations with a minor, homosexual acts, and bestiality." The category "deviational sex offense" included "sex relations with a minor, exhibitory acts, homosexual acts, and bestiality." Thus, three of the four groups comprising the second category are also included in the first category. While there are legitimate research applications of such categories, they do not form a typology.

Typologies based on theories about offenders' motivations are even more problematic because they reify the motives attributed to the offenders into categories that are readily mistaken for natural groupings with an empirical referent. A typology of rape motivations proposed by Cohen *et al.* (1971) was used by Burgess and Holmstrom (1974) to classify rapists as if each rapist had only a single, identifiable motive.

Even though motivational typologies can be refined, as has been done for rape (Groth and Burgess 1977b; Groth *et al.* 1977) and for sexual assaults against children (Groth and Burgess 1977a), they can never escape their fictitious roots. Their appeal stems from the illusion that they are explanatory and from whatever aid they may provide to the psychotherapist, whose job is too often taken to be that of rearranging motives and other linguistic constructs until the person feels better or turns elsewhere for solace. Typologies not based on motivation have been devised for sex offender research (Gebhard *et al.* 1965; Dietz 1983) and for the clinical evaluation of rapists (see Rada 1978, for a typology that is clinically useful even though it is not based on motivation and the categories are not mutually exclusive), but these are not necessarily useful for profiling.

In addition to the logical inadequacies of typologies based on motivation (namely that they have a fictitious origin and can never define a set of mutually exclusive and exhaustive categories), the use of such typologies for profiling could be crippling. This is because the correlates of a motivational class are intrapsychic (e.g., perceptions, beliefs, thoughts or feelings) rather than behavioral. Intrapsychic correlates are just what the psychotherapist needs to know, but the criminal investigator needs observable correlates so that he can go about his business of finding the offender.

In profiling, of course, much more is desired than the assignment of the offender to a category. It is desirable that he be assigned to one category in each of a number of typologies. Theoretically, the more typological "dimensions" on which he is located, the more specific one should be able to be in formulating attributional hypotheses in the fifth stage. The formulation that an offender is an organized, heterosexual, sadistic pedophile locates him in one of the 16 cells of a four-by-four table. If one holds a dimensional view of these concepts, seeing each as a spectrum between polar extremes, he has been located more or less precisely along four axes.

Stage 5: Attributional Hypotheses

In the fifth stage, the profiler uses the case-specific data, behavior, motivational hypotheses and typological hypotheses to deduce the particular attributes expected to characterize the individual who committed the offense under investigation. Some derive primarily from the typologies. For example, if the offender is hypothesized to be an organized, homosexual pedophile, one of his expected attributes is that he has an occupation (e.g., selling ice cream, teaching or pediatrics) or a hobby (e.g., coaching a boy's team, leading a boy's group or photographing children) that bring him into frequent contact with boys.

Interactions between the attributional hypothese permit greater specificity. For example, if the offende is hypothesized to be an organized heterosexual sadis it is deduced that he would enjoy engaging in an activit that would bring him into contact with pain or bloo If the case-specific data indicate that he killed a woma in her apartment in an upper lower-class neighborhoo and that he walked to her apartment, the profiler us the attributional hypothesis that the offender lives i an upper lower-class neighborhood to refine the attr butional hypothesis about his activity. Rather than t more general statement that he engages in activity th brings him into contact with pain or blood, the profil hypothesizes that the offender has worked as an amb lance driver, emergency room aid, security guard butcher's apprentice. If, in addition, the data indica that the offender gained access to his victim's apa ment under false pretenses, the profiler hypothesiz that he wore an ambulance attendant's or a securi guard's uniform, or that he showed her a false poli badge[3].

THE EXAMPLE OF LUST MURDER

Nearly a century ago, Krafft-Ebing (1965, pp. 435 436) differentiated lust murders from cases in which rape victim is killed unintentionally or in order destroy the only witness to the crime. He propos that lust murder be presumed when injuries to t genitals are found that cannot be explained by a brut attempt at intercourse, when the body has been open or when parts of the body have been removed. I classified lust murder along with other sexually sadis acts and did not distinguish between lust murder a any other type of sexually sadistic murder. In contra Allen (1969, pp. 128–129) includes under lust murd any case of deliberate killing committed for the sex sensation[4]. He points out that the first killing by a l murderer may have been unintentional, but so excit that he goes on to commit lust murders for the pleas of it. This, he suggests, may cause there to be a lo interval between the first and second murder, but w subsequent murders committed in rapid successi until the offender is arrested. Karpman (1954, pp. 12

[3] False police badges are available through mail-order advertisem in detective magazines. Such magazines are the favorite po graphy of sexual sadists (Harry, B. E., Dietz, P. E. and Hazelw R. R.: Detective magazines as sadistic pornography. Unpublis manuscript).

[4] Note that this is a motivational definition.

includes under that heading, cases of rape/murder involving no mutilation and no apparent evidence of sexual excitement from the killing per se. These are but a few of the disparate usages of the term lust murder.

Hazelwood and Douglas (1980) define lust murder as a sadistic homicide in which there has been mutilation or displacement of the breasts, rectum or genitals. They describe two types of lust murderers designated as the "organized nonsocial" and the "disorganized asocial." Their description of the characteristics differentiating the two types is sufficiently detailed to be drawn from to illustrate the conceptual model of profiling proposed here.

Suppose that in the stage of Data Assimilation the profiler notes that the mutilated body of a woman is found within view of passing cars. Investigation of the surrounding area shows that the murder was committed 50 yards away, in thick woods. The autopsy report and photographs indicate multiple, superficial, antemortem knife wounds, severe blood loss from an abdominal knife wound penetrating the inferior vena cava and postmortem mutilation of the face and amputation of the hands. The knife is not found at these scenes. Laboratory reports confirm the presence of semen in the vagina. Entomological evidence suggests that the corpse was exposed for at least 48 to 72 hours before being discovered. The victim was last seen leaving work three days prior to her body being discovered, and she was known to hitchhike home. Her body was found near a limited access highway part way between where she worked and where she lived. The murder scene can be reached by turning off the highway onto a dirt access road, driving to a clearing near the woods and walking 100 yards through the woods.

The profiler's Behavioral Reconstruction from these facts is that the offender was driving a vehicle, picked up the hitchhiking victim, headed toward her home, but turned off onto the access road. They walked into the woods together, where he tortured her with superficial stab wounds, raped her vaginally, stabbed her deeply in the abdomen, mutilated her face and amputated her hands. He departed, taking the knife and her amputated hands with him. He subsequently returned, moving her corpse closer to the road.

The profiler formulates multiple Motivational Hypotheses: The murderer was cruising for a victim and had a knife with him for that purpose when he picked up the woman. To overcome her resistance at leaving the highway and walking into the woods, he threatened her with the knife. He tortured her, drawing blood, in order to increase his sexual arousal so that he could rape her. He stabbed her in the abdomen just before, during or after penile penetration. He was excited by the mutilation, but specifically mutilated her face and amputated her hands to increase the difficulty of identifying her. He returned to move the body to a spot where it would be found because he was disappointed that the media had not yet publicized his crime. In addition to acting out his sadistic fantasy for his direct sexual gratification, he wanted to "get even" with society, have an impact on his community and punish others.

The data, behavior and motives just described allow the profiler to classify the killer as an "Organized Nonsocial" lust murderer. From the Typological Hypothesis, the profiler deduces Attributional Hypotheses. During adolescence, the murderer was a manipulator and troublemaker who had difficulty with his family, friends and authority figures and engaged in many aggressive acts. As an adult, he is self-centered, indifferent to the welfare of others and society and manipulates people with an amiable facade, though he generally dislikes people. He is methodical and cunning and lives some distance from the scenes involved in the crime. He may return to the scene to check on the progress of the investigation, may frequent police "hangouts" to overhear news of the investigation or may try to insert himself in the investigation by coming forward as an informant or to volunteer his aid in some way. He may visit the cemetery.

It should be evident that many of these Attributional Hypotheses (some of which are actually predictions of post-offense behavior) can be used to guide the investigation. All of the elements in the above example are drawn from the description by Hazelwood and Douglas (1980). These do not begin to exhaust the possible detail of attributional and other hypotheses derivable in profiling a lust murderer, but they do illustrate the stages outlined in the conceptual model.

CONCLUSION

In this paper I have proposed and described a conceptual model of the process of sex offender profiling by the FBI Academy Behavioral Science Unit. It may apply equally well (or poorly) to profiling of nonsexual offenses. This model is highly tentative, for it derives from unsystematic observation and cannot help but be heavily influenced by my own perceptions and beliefs.

The proposed model is subject to revision in the light of further observations, and it is hoped that its articulation will stimulate efforts to improve upon its shortcomings. In addition to the need for measurements of the reliability and validity of offender profiles, it is important that there be an adequate and verified anal-

ysis of how profiling is conducted. Such an analysis has the potential to improve the reliability and validity of profiling, to adapt elements of profiling to computer simulation and to provide a theoretical and conceptual basis on which profiling can be taught.

Members of the Behavioral Science Unit have written that profiling is more of an art than a science (Ault and Reese 1980; Hazelwood and Douglas 1980) Nonetheless, to the extent that the profiling process conforms to the model proposed here it may be more accurate to regard profiling as a process of logical reasoning that draws on experience, insight and judgment at each step in the process. In this sense profiling may resemble the process of clinical reasoning in medicine. Both the profiler and the physican assimilate available data, reconstruct the sequence of events, hypothesize the causal processes, assign the morbidity to typological categories and from these derive expectations about associated features, complications and future course. Thus is data assimilation like clinical examination, behavioral reconstruction like developing the patient's history, motivational hypothesis formulation like pathophysiologic analysis, typological hypothesis formulation like clinical diagnosis and attributional hypothesis formulation like clinical prognostication. The profiler, like the physician, relies heavily on his experience with similar cases, is guided by his insight where knowledge is short and exercises wise judgment if his effort is to succeed.

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CRIMINAL PERSONALITY PROFILING

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"What is to be expected . . . is an understanding not merely of the deeds, but also the doers" (Zilboorg 1968).

The study of violent crime to ascertain identifiable characteristics of the unknown offender is not a new technique. However, the work being done by the FBI's Behavioral Science Unit (BSU) represents law enforcement's entry into this area for the first time. Previously, this somewhat unique procedure had been performed primarily by individuals educated as clinical psychologists or psychiatrists. While trained in matters of the mind, they lacked the experience of conducting criminal investigations into violent crime. As a result, their profiles tended to be couched in terminology largely alien to the intended audience, the criminal investigator.

A criminal personality profile prepared by the BSU provides the investigative agency with information about the unidentified offender which differentiates him from the general population. A conscious effort is made to set forth those characteristics in a way that is easily understood and would clearly identify him or her to those who know and associate with them.

Information Provided in a Profile

While the format of the profile may vary with the individual preparing it, the provided information is essentially the same. I prefer the outline format in that it allows the reader to quickly obtain a wanted characteristic without having to read through the entire work. Regardless of the style, a profile furnished by the BSU will include most, if not all, of the following information: approximate age, sex, race, marital status, occupational level, educational background, military history, socioeconomic level, pastimes or hobbies, age and style of vehicle owned or operated, arrest history, appearance and grooming habits, residential information, victim-offender relationship and certain personality characteristics such as temperment, intelligence level, emotional adjustment, pathological behavioral characteristics and ability to interact socially and sexually.

Profiling and the Behavioral Science Unit

The BSU first began profiling on an informal basis in 1972. Our entry into this field occurred as a result of our instructional responsibilities at the FBI Academy. During courses in the behavioral sciences, faculty members would encourage students to discuss solved and unsolved cases with which they were familiar. As a result of these discussions, the instructors would note that in similar crimes, the offenders were a great deal alike. Consequently, when discussing an unsolved crime similar to one previously discussed, the faculty would provide verbal profiles to the students. The student would utilize the information upon returning to his/her investigative duties. Feedback from this exchange indicated that the assistance given was resulting in a savings of investigative manhours by properly focusing the investigation. In a few instances, it was directly responsible for solving the crime in question. Predictably, as other investigative agencies became aware of this assistance, they began submitting cases to individual faculty members for profiling. The BSU faculty are primarily instructors and, therefore, the cases would be analyzed on a time-available basis. The volume of requests grew to unanticipated proportions and in 1978, the program was further formalized when 50 Special Agents were selected from various offices within the FBI and given 80 hours of instruction to prepare them as profiling coordinators within their respective geographic regions. Since that time, agencies requesting the service submit the cases to the profiling coordinator nearest them. The coordinator in turn ensures that all necessary materials have been included and that the case lends itself to the profiling process. If all prerequisites have been met, the case is forwarded to the BSU where it is assigned for profiling. Upon completion, the profile is returned to the responsible coordinator for delivery to the requesting agency. All submitted materials are retained by the BSU for future reference.

Eventually, the caseload became overwhelming for the available staff and it became apparent that additional personnel, dedicated to profiling, were necessary. In 1983, four Special Agents (investigative profilers) were selected to understudy the BSU faculty and assume the responsibility of profiling. As a result, the backlog of cases has been reduced and more law enforcement agencies are receiving this assistance.

Procedure for Submission of Cases

Agencies desiring a criminal personality profile must submit the necessary documentation, as described later, to the profiling coordinator in the FBI office nearest the requesting agency. The material is then forwarded to the BSU where it is assigned to a profiler. Upon completion, the profile is returned to the submitting agency.

Case Criteria

Minimal criteria exist for the submission of a case for criminal personality profiling. The matter must involve a crime of violence, with the offender remaining unknown, and all investigative leads must have been exhausted. While virtually any crime possessing evidence of mental, emotional or personality aberration can be profiled, certain crimes are more susceptible to the process. Such crimes include serial rapes, lust murder (mutilation or displacement of the sexual areas of the body), serial murders, child molesting and ritualistic crimes.

Case Materials

Map: A commercially produced map is preferred to one that has been hand-drawn. The reason being that with a commercial map, the person analyzing the case is provided with an idea as to what is involved in the area (deserted, industrial, residential, schools, hospitals, etc.). If such a map is unavailable, a hand-drawn map will suffice providing a description of the area(s) involved accompanies it. The map must be notated showing the location in which the victim was approached and where the actual assault occurred. If these locations are different, the distance between them should also be provided. Any other significant locations and distances involved should also be sent forth. For instance, if the offender entered the victim's vehicle at point A, forced her to drive to point B where the assault occurred, left her there and took her car to point C where it was abandoned, all three locations would be expected to appear on the map.

Victim Statement: The interview of the victim and its documentation are the most important factors in the profiling process. Unfortunately, the person preparing the profile seldom has the opportunity to speak with the victim to obtain those facts crucial to analyzing the behavioral aspects of rape and is consequently dependent upon a third party, the investigator, to do so.

Victimology: The final documentation needed is a profile of the victim. A summary of facts known about her that would assist in determining why the rapist behaved verbally, physically and sexually as he did. Some of the essential information needed about the victim includes age, race, if she was with anyone at the time of the attack or just before, her educational level, whether she appears to be passive or aggressive in nature (Did she say or do something which caused the offender to overreact?), type of employment, and the socioeconomics of the area in which she resides. Of course, any other facts which the investigator deems appropriate should be included.

Suspects: Agencies submitting cases should not include information pertaining to suspects in the matter. If I am reviewing a case and it becomes very clear to me that seasoned investigators strongly believe that a particular person committed the crime, it is almost impossible to prepare a profile that does not strongly resemble the suspect.

Non-Profileable Cases: Not all crimes of violence lend themselves to the profiling process. There are situations or circumstances which may preclude the preparation of a valid profile. Of importance is the fact that the process is not only dependent upon the crime which has occurred and its documentation, but also upon the profiler assigned the case. In other words, what may be a difficult case or set of circumstances for one normally proficient profiler may be quite simple for another. As an example, I find it extremely difficult to profile a rape in which the offender is believed to have been under the influence of drugs, whereas this poses no problem for another member of our unit. A case in which the rapist did not speak, used minimal force and did not engage in atypical sexual activity deprives the profiler of sufficient behavior to analyze. Rapes in which the victim was rendered unconscious or because of other reasons cannot recall details are very difficult, if not impossible, to profile. A good rule of thumb seems to be that if factors are missing (excluding offender identification) which the officer needs to investigate the case, it is not susceptible to the process. It should be noted that after conversing with the investigating officers and obtaining more facts, cases which I had originally felt were not profileable were, in fact, deemed to be so. Prior to a case being returned as nonprofileable, I will study it for a consid-

erable period of time, converse with the investigating officers and discuss the matter in detail with two or three other profilers. However, the fact remains that there are cases which do not lend themselves to the profiling process.

The Profilers

When discussing criminal personality profiling, I am invariably asked what special attributes or education one must possess. My answer, quite simply, is that the successful profilers with whom I have worked possessed no particular educational degree. The qualities and attributes which I have consistently noted included investigative and research experience, common sense, intuitiveness, the ability to isolate emotions, the ability to analyze a situation and arrive at logical conclusions and the ability to think very much like the criminal.

Experience: No amount of education can replace the value of having investigated violent crime. As an investigator, one begins to collect and store data which is automatically retrieved when a new case is opened. The experienced investigator accepts nothing at face value, but questions, going beyond what appears to be the obvious. He or she does not depend on what others tell them about the crime as being truthful, but cross-checks and verifies each piece of information. This is the most significant factor differentiating the investigative profiler from the clinical psychologist or psychiatrist who prepares profiles.

During a recent conference I attended on treatment of sexual offenders, a rapist in the treatment program was presented to the audience. After he had departed, I asked the treating psychologist about the criminal history of the rapist. The clinician had not previously inquired into this area but promised to do so. Obviously he had not considered such information significant to the offender's treatment. A few minutes later, he informed the audience that the rapist had no history of arrest other than a speeding violation. I inquired as to the source of this information and was advised it was the rapist himself!

Over a period of time, an investigator develops an ability to rise above the shock of violence and to clinically move through the often gruesome, but always necessary, procedures. I am aware of certain psychologists and psychiatrists who refuse to examine photographs depicting homicide victims, yet prepare profiles for police agencies. In my opinion, this is akin to performing surgery without reviewing the patient's history.

Common Sense: While the phrase is frequently used, it has been my experience that a surprisingly large number of people do not possess this quality. This opinion is specifically addressed to those individuals who, when confronted with a situation, find it impossible to cope with unless the solution can be found in a manual or textbook. They are unable to adapt what they have experienced or learned in a classroom unless the situation encountered is exactly like the one experienced or learned. In law enforcement circles, such a person is referred to as "one who goes by the book." I am currently working on a book which deals with profiling. An overriding fear I have is that, regardless of my best efforts, some individuals will treat it as a "cookbook" and, if faced with a situation not specifically addressed in the book, will consider the entire profiling process to be of no value. It must be remembered that no two crimes or criminals are exactly alike. When one deals with human behavior, he or she finds it impossible to fit people into neat little categories. The person who has "common sense" will recognize this and will be able to project techniques from the written page or spoken word on to generally similar situations.

Intuition: Webster (1972) defines intuition as "the direct knowing or learning of something without the conscious use of reasoning—the ability to perceive or know things without conscious reasoning." Police officers refer to it as a "gut feeling." Like common sense, it is not something which can be learned in a classroom or from a book. Realistically, it is probably not a trait that a person is born with either, but rather an ability born from experienced, but forgotten, occurrences. Regardless of its origins, it is a fact that some individuals possess an intuitive ability that is extremely valuable in the profiling process.

Recently, a prosecutor traveled to the FBI Academy for consultation with myself and another member of the Behavioral Science Unit, John Douglas. He was about to prosecute a man for the rape/murder of an 18-year-old paraplegic girl. The defendant had been arrested after confessing to the crime but had refused to discuss what had motivated the murder. The prosecutor was justifiably concerned that, unless he had an understanding of why the crime occurred, he would have difficulty in presenting knowledgeable and convincing arguments. After reviewing the case materials and discussing the details with him and his associate, we felt confident in reconstructing the manner in which the crime occurred and the motivational factors involved. As he was about to leave, John Douglas stated that he had "a feeling" that the date of the murder might have some significance to the killer and suggested tht the attorney pursue this possibility. It was later learned that the date of the murder was the anniversary of the day on which the murderer was forced to leave his mother's home. The mother was a

very domineering person who had engaged in incest with the young man over a period of years. The defendant despised her but was emotionally dependent on her The prosecutor later advised that the knowledge of this factor played a large role in the successful prosecution of the offender.

Isolation of Affect: The successful profiler is one that is able to isolate his personal feelings about the crime, the criminal and the victim.

> A 16-year-old girl was kidnapped by two young men and, over a 6-hour period of time, was subjected to sexual assault and physical torture. Her head and pubic hair had been pulled out or burned and she had been severely beaten. She survived but underwent extensive hospitalization and therapy.

As presented above, the case is somewhat clinical; however, the materials submitted for study consisted of graphic medical records and photographs and a detailed statement from the victim. As a parent of teenage children, a law enforcement officer and a member of society, I am outraged at the injustice of such an attack on a child. As a profiler, however, I cannot allow these feelings to interfere with the task at hand.

Personal feelings about the criminal must also be isolated. For instance, in the crime described above, one not knowledgeable about such matters might assume that the responsible persons are insane, have extensive arrest histories and are unable to function within society. In fact, there is a very real possibility that none of these assumptions is correct. When preparing a profile, one should attempt to describe the offender as those who know him would describe him. The reader is reminded of recent and infamous serial murderers who were perfectly rational and fully functioning individuals with no history of arrest or mental illness. Kenneth Bianci (Hillside Strangler), John Wayne Gacy, Theodore Bundy and Wayne Williams (Atlanta Murders), to mention a few.

Finally, the profiler must isolate his feelings about the victim. In many instances, the victims of sexual assault and/or homicide are what we refer to as "high-risk" victims. That is to say, the victim may have been particularly vulnerable because they were prostitutes, involved in drug-related activities, hitchhiking or runaways. If the profiler allows personal feelings about the victim to enter the evaluation, it will seriously impair the process.

Analytical Logic: The ability to study a situation and arrive at logical conclusions is not one that all individuals possess. In profiling, one must make conclusions based upon what has been observed, heard or read. A great deal of the mystique surrounding the art of profiling disappears when one realizes that a large amount of information provided in a profile is analytically and logically arrived at. There are two types of characteristics provided in a profile: primary and secondary. A primary characteristic is determined directly from the manner in which the crime was committed, while the secondary characteristic is derived from the primary. Age of the offender is a primary characteristic arrived at by recognizing behavioral evidence of maturity. Let's assume that I have stated the offender is between 45 and 50 years old (primary); then it is quite reasonable for me to indicate that he is a military veteran (secondary) inasmuch as practically all males within that age range served in the armed forces due to the draft. Another example of the application of logic might involve a rapist which is believed, for one reason or another, to be employed in a white-collar occupation (primary). It is then logical to assume that he will operate a vehicle less than 5 years of age (secondary).

Think like the Criminal:

> In a large metropolitan area, a series of rapes had plagued the police over a period of months. In each instance, the rapist had controlled his victim through threats and intimidation. One evening a hospital orderly went off duty at midnight and happened upon a male beating a nurse in attempt to rape her The orderly went to her rescue and subdued the attacker until the police arrived. Predictably, he received much attention from the news media and received a citation for bravery from the city. Shortly thereafter, the orderly was arrested for the series of rapes mentioned earlier. During interrogation, he was asked why he had rescued the nurse when he, in fact, was guilty of similar offenses. He became indignant and advised the officers that they were wrong. He would never "hurt" a woman.

This offender equated "hurt" with physical trauma. The point is that intent becomes clear only if we attempt to view the crime from the motivational standpoint of the criminal (Hazelwood 1983). The ability to observe the crime from the perspective of the criminal is the result of having dealt with violent crime and criminals over a period of years. It is not something that can be learned in a classroom. The profiler must forget that he or she is a parent, a spouse or a law enforcement officer and temporarily assume the role of the criminal. Then he or she must begin to ask questions about the crime. "Why would I continue to beat the victim after all resistance had ended?" "Why wouldn't I react more violently after the victim bit me?" To assume this role is not an easy task in that violence is what we, as law enforcement officers, are charged with preventing.

I recall a murder case in which John Douglas and I were providing on-site consultation. It involved the kidnap and murder of a 12-year-old girl who was

found after 5 days. After we had presented the profile to the officers investigating the matter, a clinical psychologist in attendance stated that we were, in effect, describing a paranoid schizophrenic We concurred with that assessment and he then asked how we could be so comfortable with that evaluation after simply studying the crime-scene data. We explained that we attempt to re-enact the crime, viewing it as the murderer did and to reason as he did. He then asked if we would undergo a personality inventory, responding as we believed the offender would if he were in custody. We agreed, and independently took the examination. After reviewing the results, the psychologists advised that our responses would have been evaluated as those of a paranoid schizophrenic. To be able to think like a criminal is an important attribute in profiling.

The Profiling Process: A criminal personality profile is a series of subjective opinions about the unknown individual(s) responsible for a crime or series of crimes. The process in arriving at these opinions is quite difficult to articulate in that the final product is largely dependent on common sense, intuition and the experience of the profiler as earlier discussed. In this brief space, I will merely attempt to acquaint the reader with the process I utilize when profiling a rape case.

In preparing a profile, I utilize three basic steps: (1) Determine from the victim what behavior was exhibited by the rapist. (2) Analyze that behavior in an attempt to ascertain the motivation underlying the assault, and (3) Set forth the characteristics of the individual who would commit the crime (in such a manner) to satisfy the motivational factor indicated by behavior (Hazelwood 1983).

It has been my experience to observe that similar cases committed for similar reasons are generally perpetrated by similar offenders. In other words, what = why = who. Given a rape which occurred in Houston, Texas, I can produce a rape which occurred in Arlington, Virginia, that is so similar in nature that one might assume the same individual was responsible for both crimes. The explanation for this is really quite simple. The crimes are similar because the underlying motivation is basically the same; therefore, it is logical to assume that the offenders will be as generally similar as their crimes. Now let's examine each step individually.

Determine Offender Behavior: It is the offender's behavior, exhibited during the commission of a crime, that is studied by the profiler. In sexual assaults, the victim may be able to provide information on three forms of offender behavior: verbal, sexual and physical (force). She can advise what the offender said or demanded that she say, the type and sequence of sexual acts that were performed and the amount of physical force used by the offender. Provided with this information, it is probable that the profiler can determine the underlying and nonsexual reason for the assault.

Analyze the Behavior: It is at this step that one studies and evaluates the verbal, sexual and physical behavior of the rapist. The purpose being to determine the true motivation for the sexual assault. As Groth (1979) points out "... rape is, in fact, serving primarily nonsexual needs." One should examine the verbal behavior for indications of hostility, anger, a need for affection, concern or politeness, among other things. The type and sequence of sexual behavior should be analyzed, seeking to determine if there was offender intent to degrade, involve or punish the victim. Finally, the amount of physical force should be looked at. At what point did the rapist apply force, was it to intimidate or punish, and did he continue to use force when resistance had ceased? It must be recognized that the motivation for the crime is exhibited through the rapist's behavior.

Prepare the Profile: Once an assumption has been made as to what motivated the crime, the rapist can then be profiled. The manner in which an individual behaves within his various environments portrays the type of person he or she is. Opinions are formed about a person's self-esteem, educational level, ability to negotiate interpersonal relationships and goals in life by the manner in which the individual behaves.

A Word of Caution about Profiling

I begin lectures on this subject by advising my students that criminal personality profiling is an art and not a science. It is simply another investigative tool to assist in the investigation of violent crime. It is not intended to surplant any other investigative step and, in fact, we prefer not to prepare profiles until all investigative procedures have been accomplished and the case is at a standstill. If one depends solely upon a profile to solve his or her case, that person will have acted irresponsibly and will find it counterproductive to the goal of crime solving.

While profiles have directly led to the solving of a case, this is the exception rather than the rule and to expect this will lead to failure in most cases. Rather, a profile provides assistance by focusing the investigation of suspects to those matching the characteristics set forth. It should also be made clear that the profile may fit more than one individual, even within the same neighborhood. In a recent rape/homicide that I worked on, the profile fit three different people residing in close proximity to the victim. When the police confronted me with this dilemma, I could only advise them that the killer was either one of the three *or* someone else

like them. They were not particularly pleased with that piece of information but understood that profiling is intended to identify a personality type, not a person.

As with any other human endeavor, failures will occasionally occur and this should not detract from the process. When being interviewed by Porter who wrote the article entitled "The Mind Hunters" for the April 1983 issue of *Psychology Today*, I brought to his attention that I had prepared a particularly erroneous profile involving the assault of a mother and the shooting of her three-month-old baby. After the responsible person had been arrested and confessed, a comparison of my profile with the arrested offender revealed I had been correct in only two areas. I made mention of that case then, and do so now, to ensure that the reader does not misinterpret the value of this very subjective process. Human behavior is much too complex to neatly label; to suggest that we in the BSU have done so, or are attempting to do so, would not be true. Profilers are not blessed with a sixth sense, nor do they have a crystal ball which provides them with mystical powers. We are encumbered with the same human frailties as everyone else. We have simply had the opportunity to observe a very large number of violent crimes and to assimilate that experience into our work. No one is more enthusiastic about criminal personality profiling than I am. However, I am the first to acknowledge that proven investigative procedures solve crimes, not profiling.

SUMMARY

Criminal personality profiling was initiated in the FBI Academy's Behavioral Science Unit on an informal basis in 1972. The program was formalized in 1978 and in 1983, additional investigative profilers were assigned and dedicated to the task.

The criteria for case submission are that it involve a crime or series of crimes of violence which are unsolved and that all investigative leads have been exhausted.

Successful profilers are experienced in criminal investigations and research, possess common sense, intuition and are able to isolate their feelings about the crime, the criminal and the victim. They have the ability to analytically evaluate the behavior exhibited in a crime and to think very much like the criminal.

When analyzing a crime for profiling purposes, it is necessary to evaluate what occurred in order to determine the underlying motivation for the crime. One is then able to construct a profile of the person who would have committed such a crime for such a reason. In sexual assaults, the verbal, sexual and physical behavior of the offender is evaluated.

Criminal personality profiling should be used as an augmentation to proven investigative techniques and must not be allowed to replace those techniques. To do so would be counterproductive to our common goal of identifying the unknown offender.

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SUBJECT INDEX